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TRACHEAL ORGAN CULTURE AS AIR POLLUTION DAMAGE INDICATOR

by

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16. ABSTRACT

This report presents the results of a study conducted to determine the effects of various energy-related effluents on respiratory tract epithelial tissue. Measurement of mucoiliary activity and characterisation of the morphological alterations induced by such effluents was carried out in hamster tracheal organ culture. Different combinations of in vivo and in vitro exposure and/or maintenance were used to determine the relationship between in vivo exposure danger and adverse effects observed in organ tissue.

The pollutants assed included particulate effluents from mobile and stationary sources of both conventional and advanced energy processes. Included were fly ash (from coal-fired and oil-fired sources), cigarette smoke condensate, and diesel fuel exhaust extract, with benzo(a)pyrene serving as a positive control.

Both acute (72-hr) and long-term 14-day studies permitted assessment of tissue specific effects. In addition to acute and long-term toxicity studies, testing was conducted to determine the effects of selected particulate effluents on the pathogenesis of viral infections.

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FOREWORD

The many benefits of our modern, developing, industrial society are accompanied by certain hazards. Careful assessment of the relative risk of existing and new man-made environmental hazards is necessary for the establishment of sound regulatory policy. These regulations serve to enhance the quality of our environment in order to promote the public health and welfare and the productive capacity of our Nation's population.

The Health Effects Research Laboratory, Research Triangle Park, conducts a coordinated environmental health research program in toxicology, epidemiology, and clinical studies using human volunteer subjects. These studies address problems in air pollution, non-ionizing radiation, environmental carcinogenesis and the toxicology of pesticides as well as other chemical pollutants. The Laboratory participates in the development and revision of air quality criteria documents on pollutants for which national ambient air quality standards exist or are proposed, provides the data for registration of new pesticides or proposed suspension of those already in use, conducts research on hazardous and toxic materials, and is primarily responsible for providing the health basis for non-ionizing radiation standards. Direct support to the regulatory function of the Agency is provided in the form of expert testimony and preparation of affidavits as well as expert advice to the Administrator to assure the adequacy of health care and surveillance of persons having suffered imminent and substantial endangerment of their health.

Energy processes such as the burning of coal and oil produce effluents which can be linked to respiratory damage in humans. Fly ash, a particulate byproduct of both coal and oil combustion, is present in the ambient air in large concentrations.

Since exposure to fly ash and other airborne particulates is unavoidable, the study of their effects on human health is essential.

F.G. Hueter, Ph.D.
Director
Health Effects Research Laboratory

ABSTRACT

This report presents the results of a study conducted to determine the effects of various energy-related effluents on respiratory tract epithelial tissue. Measurement of mucociliary activity and characterization of the morphological alterations induced by such effluents was carried out in hamster tracheal organ culture. Different combinations of *in vivo* and *in vitro* exposure and/or maintenance were used to determine the relationship between *in vivo* exposure danger and adverse effects observed in organ tissue.

The pollutants assayed included particulate effluents from mobile and stationary sources of both conventional and advanced energy processes. Included were fly ash (from coal-fired and oil-fired sources), cigarette smoke condensate, and diesel fuel exhaust extract, with benzo(a)pyrene serving as a positive control.

Both acute (72-hr) and long-term (14-day) studies permitted assessment of tissue specific effects. In addition to acute and long-term toxicity studies, testing was conducted to determine the effects of selected particulate effluents on the pathogenesis of viral infections.

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LIST OF ABBREVIATIONS AND SYMBOLS

ABBREVIATIONS

ip	-- intraperitoneal
BP	-- benzo(a)pyrene
bpm	-- beats per minute
BSS	-- balanced salt solution
CSC	-- cigarette smoke condensate
DFEE	-- diesel fuel exhaust extract
DMSO	-- dimethylsulfoxide
ESP	-- electrostatic precipitator
HAD_{50}	-- 50 percent endpoint titration determined by hemadsorption
HID	-- hamster inhaled dose
PAS	-- periodic acid Schiff
RH	-- relative humidity
SEM	-- scanning electron microscopy

SYMBOLS

AS	-- Arsenic
Cu	-- Copper
Nb	-- Niobium
Pb	-- Lead
Rb	-- Rubidium
Sr	-- Strontium
Ti	-- Titanium
V	-- Vanadium
Y	-- Yttrium
Zn	-- Zinc
Zr	-- Zirconium

SECTION 1

INTRODUCTION

Air pollution exposure is linked to increased frequency and severity of acute respiratory disease. Certain exposure conditions to environmental pollutants may impair the function of important defense mechanisms of the respiratory epithelium. Pathologic consequences of the pollution exposure could therefore, be manifested as a shift in normal cell populations, cytostructural abnormalities of cells and organelles, and changes in repair processes. These adverse effects could render the tracheobronchial epithelium more susceptible to respiratory infections and vulnerable to non-infectious pollutant agents. To be environmentally acceptable, conventional and advanced energy processes must meet several health and safety regulations. It has therefore become increasingly important to examine any health effects that may result from fugitive emissions from these processes.

The overall goal of this research project was to provide health effects data associated with a variety of hazardous pollutants. The studies were performed using organ culture techniques developed in our laboratories for maintaining differentiated respiratory tract epithelial tissue from suckling and adult hamster trachea. The specific objective was to compare the effects of conventional and advanced energy processes on tracheal mucociliary transport parameters. Both *in vitro* and *in vivo* inhalation exposures were conducted. In this manner the validity of the *in vitro* test was assessed for suitability as a rapid screening model. Dose-response studies of acute and intermittent exposures were conducted and recovery from any adverse effects of this treatment was examined. This organ culture model, which isolates air pollutant effects from secondary influences such as inflammation, immune and humoral responses, permitted studies on the mechansims of viral infection during and after exposure to these pollutants. The specific pollutants used in this study were fly ash, cigarette smoke condensate (CSC) and diesel fuel exhaust extract (DFEE). Their effects on organ culture were measured by cilia beating frequency using an electronic stroboscope, sequential morphological and cytopathologic changes of the tracheal epithelium using light and scanning electron microscopy and influenza virus replication.

During the initial phase of the program hamster tracheal tissue was used to study the effects of H_2SO_4 mist, carbon particles, and mixtures of the two on cilia beating frequency and morphological alterations of respiratory epithelium. Different combinations of *in vivo* and *in vitro* exposure and/or maintenance were used to determine the relationship between *in vivo* exposure damage and recovery and effects observed in organ culture. These studies were summarized in Annual IITRI Report L6080-12 (October 1977).

The results of these studies were summarized in quarterly progress and annual reports, submitted to the EPA Project Officer. Publications resulting from these studies are:

Technique for determining ciliary activity in hamster tracheal organ culture. In: Tissue Culture Assoc. Manual: Techniques, Methods and Procedures for Cell, Tissue and Organ Culture. V. J. Evans, V. P. Perry and M. M. Vincent (eds.) 4, 871, 1978. L. J. Schiff, M. M. Byrne and W. T. Brown.

Cytotoxic effects of sulfuric acid and carbon-particle mixtures on hamster tracheal epithelium. Proc. Annual Meeting of the Tissue Culture Assoc., Inc. Denver, CO, June 4-8, 1978. L. J. Schiff, M. M. Byrne, J. D. Fenters, R. Ehrlich, J. Graham and D. E. Gardner.

Scanning electron microscopic study of developing hamster tracheal epithelium in organ culture. Proc. Annual Meeting of the Tissue Culture Assoc., Inc. Denver, CO, June 4-8, 1978. L. J. Schiff, K. V. Ketels, M. M. Bryne, R. Ehrlich and J. Graham.

Cytotoxic effects of sulfuric acid mist, carbon particulates, and their mixtures of hamster tracheal epithelium. Environ. Res. 19, 339, 1979. L. J. Schiff, M. M. Byrne, J. D. Fenters, J. A. Graham and D. E. Gardner.

A scanning electron microscope study of developing hamster tracheal epithelium in organ culture. Differentiation 15, 49, 1979. L. J. Schiff, M. M. Byrne, K. V. Ketels, W. T. Brown and J. A. Graham.

Comparison of the effects of coal-fired fly ash and oil-fired fly ash on hamster trachea in organ culture. Proc. Annual Meeting of the Tissue Culture Assoc. St. Louis, MO, June 1-5, 1980. L. J. Schiff, M. M. Byrne, S. F. Elliott and J. A. Graham.

SECTION 2

CONCLUSIONS

Evaluation of the health effects of potential environmental pollutants has been traditionally based on chronic bioassays in rodents. These bioassays are complex, requiring controls of many variables for many years. It was therefore, the purpose of these studies to utilize *in vitro* methods in order to provide a more rapid, sensitive and relevant test system for screening a variety of hazardous pollutants. Tracheal organ culture methods developed in our laboratory were used to investigate both short- and long-term toxic effects of environmental pollutants, and to determine the effects of selected particulate effluents on the pathogenesis of viral infections. Results of the studies indicated that (1) with the majority of pollutants tested, suckling hamster trachea in organ culture was more sensitive compared to ring cultures from adult hamsters, (2) cilia beating frequency and cytopathology alone were not adequate as pollution damage indicators and should be used in conjunction with light microscopy and SEM examinations, and (3) infection of trachea cultures with influenza virus before *in vitro* exposure to particulate pollutants (e.g., coal-fired fly ash) produced a greater mucociliary epithelial response than the pollutant alone.

These *in vitro* systems were used to evaluate the potential health effects of energy-related effluents produced by fossil fuel and oil combustion. The toxic effects of coal-fired fly ash and oil-fired fly ash on tracheal mucosal epithelium and the relationship between fly ash exposure and toxicity were assessed in hamster tracheal organ culture. The results of these studies showed (1) that hamster tracheal organ cultures can be used to study acute effects of fly ash on respiratory ciliated epithelium, (2) the toxic effects produced by the two fly ash species are the result of different mechanisms of inducing injury, (3) toxicity of oil-fired fly ash is directly proportional to the concentration and duration of exposure. Morphological observations indicated that cytotoxic concentrations of oil-fired fly ash are below the level that produced ciliostasis, and (4) the transformation of tracheobronchial epithelium to epidermoid metaplasia by coal-fired fly to be similar in response to mechanical stimuli, effects of carcinogens, vitamin A deficiency and nutritional influences *in vitro*. Results also indicated a correlation between fly ash-induced changes in tracheobronchial epithelium of hamsters exposed *in vivo* (2 mg/m^3) and effects observed in hamster tracheal epithelium exposed in organ culture (5-10 µg/ml).

The organ culture assay permitted toxicity ranking of the pollutants tested. In decreasing toxicity they were, oil-fired fly ash>cigarette smoke condensate>coal-fired fly ash>diesel fuel exhaust extract.

Studies on the effect of influenza A/PR-8 virus on the expression of fly ash damage have shown that tracheal explants infected 24 hr prior to coal-fired fly ash exposure produce a significantly greater response than in explants exposed immediately after infection. These findings may have bearing on the hypothesis that influenza may stimulate the transformation of tracheobronchial epithelium to epidermoid metaplasia by (1) altering the permeability of target cells, so that the penetration of the fly ash is facilitated, (2) increasing the mitotic activity of the cells providing a favorable condition for an attack by the aromatic hydrocarbons and trace elements that constitute the soluble fraction of the fly ash, or (3) interference with the detoxication mechanisms of the target cells, leading to an increased persistance of the aromatic hydrocarbons.

Environmental exposure to airborne particulates has been linked to chronic respiratory disease, such as chronic bronchitis. Upon inhalation pollutants are deposited to a large extent in the trachea where they are removed by mucociliary mechanisms, or phagocytized. The effects of the various pollutants on cells of the tracheobronchial epithelium were in many instances complex. Each of the real-world particulate pollutants assayed produced a unique response, probably related to chemical composition and size of the particles. It is recognized that smaller particles are phagocytized by mucosal cells while the larger particles provoke hyperplastic and metaplastic changes. One might speculate that exposure to the particulate pollutants affect the structure and functional continuity of the epithelium, thereby contributing to chronic bronchitis and may act as cocarcinogens.

SECTION 3

RECOMMENDATIONS

The results of our studies have provided health effects data on several air pollutants associated with the emerging energy technologies. Organ cultures of hamster trachea consisting in part of mucociliary epithelium have permitted studies of the effects of toxic agents on this tissue. The ring and whole tracheal organ culture preparations have been well suited to evaluate a number of parameters on viable explants as well as fixed tissue. These cultures were maintained for weeks and monitored while being exposed for a specific period under completely defined conditions. The ability to prepare a large number of tracheal cultures has permitted multiple replicate exposures at each experimental point and a wide range of concentrations. Our results demonstrated that changes in any single parameter are not sufficient indications of the toxic effects of exposure to a test substance and therefore we recommend that cilia beating frequency data be correlated with morphological and biochemical parameters.

The increasing number of environmental pollutants being emitted into our environment and the backlog of potentially hazardous chemicals used in industry make a rapid, reproducible and relatively inexpensive test system for evaluating toxicity a pressing need. Hamster tracheal organ cultures provide the necessary criteria to facilitate identification of these toxic agents. Additional investigations are therefore needed to determine the response of mammalian respiratory tract tissue to a variety of complex mixtures.

The studies should be expanded by using organ cultures of tracheal epithelium and heterotopic tracheal grafts as a bioassay for respiratory tract carcinogenicity to evaluate environmental pollutants as potential carcinogens. The criteria for assessment would include histologic and cytologic changes, DNA synthesis and tumor production. In this way, the questions concerning the interrelationships between differentiation, cell population kinetics, and various stages in carcinogenesis could be possibly clarified.

SECTION 4

EXPERIMENTAL PROCEDURES

TEST ANIMALS

The studies described in this report utilized 2- to 4-day old and 4- to 6-week-old Syrian golden hamsters purchased from Engle Laboratory Animals, Inc., Farmerburg, IN and ARS/Sprague Dawley, the Mogul Corp., Madison, WI. The Syrian hamster was our choice of experimental animal because it has a low incidence of respiratory infections[1]. The histologic features of the hamster respiratory epithelium resembles that of man adequately enough to permit valid interpretation of results in terms of the human respiratory tract[2].

For tracheal grafting experiments syngenic hamsters were required. The carcinogen-susceptible F1D hybrid, B10 F1D Alexander, recently developed by Bio-Research Consultants, Inc., Cambridge, MA and TELACO, Bar Harbor, ME from B10 15.16 sires and B10 87.20 dams were used (Van Dongen, personal communication).

TEST COMPOUNDS

Materials which were assayed include fossil fuel fly ash (coal-fired and oil-fired), BP, cigarette smoke condensate and diesel fuel exhaust extract. Coal-fired fly ash used in these studies were supplied by the EPA and characterized by Oak Ridge National Laboratory[3]. The facility sampled was a coal-fueled power plant using Eastern Kentucky coal. The sample, consisting of ESP hopper fly ash, was collected from the last plate of the ESP. The material as we received it was designated as CPPE-1 powder sample EP-4. The median geometric diameter of the particles was 1.8 μm. Major elements present were As, Cu, Nb, Pb, Rb, Sr, Ti, V, Y, Zn and Zr. Seven polycyclic organic compounds (BP, chrysene, benz(a)anthracene, pyrene, phenanthrene, fluoranthene and anthracene) were also identified.

The oil-fired fly ash material was received from Paul Cavanaugh, Southern Research Institute, an EPA contractor, and is an electrostatic precipitator fly ash sample collected from an oil-fired power plant. The data concerning characterization and composition of this fly ash sample were not provided to IITRI.

The CSC and DFEE samples were also supplied by EPA. Detailed characterization of these particulates are being made but results are not yet available.

Most of the materials that were assayed were freshly prepared the day of use since compound stability was unknown. Control and test agents were stored according to supplier directions regarding temperature, humidity and protection from light. If specific directions were not received, compounds were stored at 2°C in the dark.

TRACHEAL EXPLANT PREPARATION

Preparation of Tracheal Rings and Cultivation Method

Four-week-old Syrian golden hamsters were anesthetized by injecting 0.5 cc Brevital sodium into the abdominal region. Animals were immediately opened from the middle of the sternum to the larynx. The trachea was exposed and the surrounding membranes aseptically teased away with fine forceps. The trachea was gently lifted and a forceps was slid underneath to free it from the esophagus and to facilitate cleaning. After removing remaining membrane, the trachea was excised, cutting just above the larynx and just above the bifurcation. The trachea was briefly rinsed in Hanks' BSS and cleaned of remaining membrane. The larynx-end of the trachea was grasped with a sterile curved hemostat which was laid against the edge of the dish at an acute angle with the trachea held over the dish. Each trachea was opened from the larynx to the corina along the membraneous dorsal wall. After grasping the loose end with sterile forceps, the trachea was gently stretched to slightly separate the white cartilage rings. With a sterile scalpel the trachea was cut into rings approximately 1 mm thick. Approximately 10 to 12 tracheal rings were obtained from each animal.

Tracheal rings were washed in Hanks' BSS and placed on a section cross-hatched with a scalpel blade in 35 x 10 mm unwettable plastic petri dishes containing 0.7 ml of CMRL 1066 medium supplemented with 0.2% bovine albumin, penicillin (250 U/ml), and streptomycin (250 µg/ml). The rings were incubated for 24 hr at 37°C in 5% CO_2 atmosphere and 90% relative humidity. This initial incubation period allows the rings to adjust to the external environment and ensures that normal beating frequency is restored before the test substance is applied.

Preparation of Whole Tracheal Explants

Whole tracheal organ cultures from 4-6 day-old hamsters were prepared as described by Schiff and Shefner[4]. Two explants were placed lumen side up on the surface of a silk screen supported by a stainless-steel grid positioned over the center well of an organ culture dish. Culture medium described for the ring cultures was added to each dish until its surface was level with the epithelium of the tracheal explant. All cultures were incubated at 37°C in a humidified atmosphere. Culture medium was replaced three times a week.

Examination of Tracheal Cultures--

Immediately following removal from the hamster, all tracheal explants were examined using an inverted Nikon microscope at 200 X for normal ciliary activity and evidence of cytopathology.

Ciliary measurements--Cytotoxicity resulting from exposure to test compounds was expressed as alterations in ciliary activity and cytopathology. Cilia beating frequency for each tracheal explant was measured at four separate areas on the lumen using an electronic stroboscope and their average was recorded as beats per minute[5]. The cilia beating frequency of whole trachea was observed by focusing on the mucosa through the explant. After the initial 24 hr incubation, the baseline cilia beating frequency for each explant was determined.

Epithelial damage--The percentage of normal epithelium (smooth luminal surface with beating cilia) was recorded for each explant using an inverted phase microscope at a magnification of 100 X.

Histology, histochemistry and SEM--Immediately after removal from the animal or at designated times during experiments, explants were fixed in neutral buffered formalin, dehydrated, cleared and embedded in paraffin, and sections stained with hematoxylin and eosin. Paraffin sections were also stained with an alcian blue at pH 2.5-PAS sequence with distinguished blue- or purple-stained acidic mucosubstance from red-colored neutral mucosaccharide.

Trachea specimens for SEM were fixed with Karnovsky's paraformaldehyde-glutaraldehyde phosphate-buffered fixative. Preparation for SEM was performed as described previously[6]. After critical point drying and coating with gold, the specimens were examined with a Mark II Stereoscan SEM.

Statistical analysis--To eliminate animal-to-animal variability, each treatment group and control were assigned two ring explants from each hamster. Experimental error was further reduced by using the same ring to determine the differences between the baseline cilia beating frequency and the beating frequency at different times following exposure. Two-way analysis of variance was used to test the hypothesis of no treatment differences for the time periods studied. Duncan's New Multiple-Range Test and Chi Square Distribution Test was used to determine patterns of significant treatment differences. In statistical comparisons, $p<0.05$ was chosen for accepting or rejecting differences between groups as significant.

POLLUTANT EXPOSURE

Fly Ash Exposure: *In Vivo*

Hamsters were exposed to fly ash (CPPE-1 powder sample) in 432-liter-capacity plastic chamber maintained at 25-29°C and 30-55% RH. Homogeneous distribution to the experimental environment was assured by entering and exhausting the test compounds on opposite ends of the chamber and by continuously operating a small fan to provide proper distribution. The

compressed air supplied to the exposure chamber for dilution of dissemination of the test compounds was passed through appropriate filter systems to dry the air and to remove all traces of oil and particles.

Mass concentration of the particle aerosol in the exposure chamber was monitored using a Model JM-7000 aerosol, smoke and dust photometer (Phoenix Precision Instruments) which operates on the principal of forward light-scattering. The photometer measures a relative aerosol concentration by detecting the intensity of light scattered by particle samples through the scattering chamber. Intensity of the scattered light is proportional to the mass concentration of the suspended particles. The reading from the photometer depends on particle size, shape, refractive index and size heterodispersity. The photometer was calibrated for the particular light-scattering qualities of each material by generating aerosols of different mass concentrations and collecting samples for gravimetric analysis on 0.1 μm Nuclepore filters (Nuclepore Corp., Pleasantown, CA). The average mass concentration was then plotted against the average of reading obtained with the photometer during the sample collection interval.

In addition, a condensation nuclei counter (Type CN Small Particle Detector; Gardner Assocaites, Inc.) was used for periodic determination of the aerosol concentration. The two instruments were used to assure that the mass concentration was maintained in the required range during the entire exposure period. Actual mass concentration was calculated from the gravimetric samples collected and the total volume of air sampled during the entire 3-hr exposure period. The desired mass concentration for the trials was 2000 $\mu g/m^3$ of the CPPE-1 particles; the actual concentration was 1987 $\mu g/m^3$ (SE 76 $\mu g/m^3$).

Fly Ash Exposure: *In Vitro*

Fly ash (coal-fired and oil-fired) was suspended in CMRL 1066 medium at 5, 10, 50, 100, 250 and 500 μg/ml. Tracheal ring explants were exposed to fly ash in 60 x 15 mm petri dishes. The culture dishes were placed in a controlled atmosphere chamber and maintained in 5% CO_2 and balanced air. The chamber then was placed on a platform which rocked at 10 cycles/minute, causing the medium containing the test agents to flow continuously over the epithelial surface. The fly ash was added to the tracheal organ cultures and explants exposed for 1 or 3 hr/day, 5 days/week for 10 days using the rocker platform technique. Following exposure, the explants were washed, transferred to the appropriate culture dish, and maintained at 37°C in a humid 5% CO_2-air environment in CMRL 1066 medium containing 0.2% bovine albumin and antibiotics.

VIRUS INFECTION

Virus Preparation

The influenza A/PR-8/34 (HON1) virus stock was prepared from hamsters following intranasal inoculation. A 20% lung suspension in phosphate-buffered saline was made, centrifuged at a low speed, and the supernatant dispensed in sealed ampoules and stored at -70°C. The virus pool had a titer of $10^{4.2} HAD_{50}/ml$.

Virus Infection of Hamsters--Male golden Syrian hamsters 4-6 weeks-old and weighing approximately 70 gm each were used. The animals were lightly anesthetized with ether and then inoculated intranasally with 0.1 ml of the virus suspension containing $10^2 HAD_{50}$ of influenza A/PR-8 virus. Control hamsters were given only the virus diluent. One hour after inoculation, one group of animals was anesthetized, tracheas excised and organ cultures prepared by the technique previously described. Another group of animals was held for *in vivo* studies. At various intervals the hamsters were anesthetized and tracheal explants prepared.

Infectious Challenge: *In Vivo*

Infectious Challenge--The hamster were infected with airborne influenza A/PR-8 virus by the respiratory route. Infectious aerosol challenge was conducted in a 350-liter plastic aerosol chamber (60x60x95-cm) installed within a microbiological safety cabinet. A continuous flow DeVilbiss atomizer (Model 84) was used to disseminate the influenza virus with particles of 1 to 5 μm MMD by means of filtered compressed air. For the challenge, hamsters were placed in the aerosol chamber and exposed for 10 or 15 min to 100 HAD_{50}/ 0.1 ml of influenza virus. After the challenge, the hamsters were removed from the chamber and held in an isolated clean air animal room.

Virus Inoculation: *In Vitro*

Tracheal rings showing strong ciliary activity 24 hr after planting were used for the viral infectivity experiments. Culture medium in dishes containing tracheal rings was withdrawn without removing the explants. Tracheal explants were inoculated with 0.2 ml of the virus suspension containing $10^{2,5}$, $10^{2.0}$, $10^{1.5}$, or $10^{1.0} HAD_{50}$ hamster-adapted A/PR-8 influenza virus. After 1 hr adsorption at 37°C, each explant was aseptically removed from the dish, washed with Hanks' balanced salt solution, and placed in a second dish. At that time 0.7 ml of medium was added to ring cultures and approximately 1 ml to whole tracheal cultures, and the dishes incubated at 37°C in 5% CO_2.

For studies to determine the effect of virus concentration on whole tracheal explants, duplicate experiments were performed with explants from four animals. In every experiment, two organ culture dishes each containing two explants were inoculated with a three-fold dilution of the virus suspension. Explants were infected with $10^{2.5}$, $10^{2.0}$, $10^{1.5}$ or $10^{1.0} HAD_{50}$ hamster-adapted A/PR-8 influenza virus. After 1 hr adsorption at 37°C in 35 mm culture dishes, the explants were washed and transferred back to organ culture dishes and incubated at 37°C in 5% CO_2-air.

Virus Titration

Culture medium was removed, at designated time periods, from infected and control organ cultures for titration and replaced with fresh medium. The virus was titered in Vero (African green monkey kidney) cells using quadruplicate wells (96 well microtest plate) per dilution . Serial tenfold dilutions of each sample were made and each well inoculated with 0.1 ml.

Cultures were incubated at 37°C and on the fourth day, the culture medium was removed and 0.1 ml of 0.4% guinea pig red-blood-cell suspension added to each well. After 30 min incubation at room temperature, the cells were washed and observed for hemadsorption. The 50% endpoint titration was determined by the hemadsorption dose (HAD_{50}) and calculated by the method of Reed and Muench[7].

SALMONELLA MUTAGENICITY ASSAY

Five mutant strains of *Salmonella typhimurium*, TA98, TA100, TA1535, TA1537 and TA1538 received from Dr. Bruce Ames at the University of California, Berkeley were used. Stock cultures of each strain were grown and checked to confirm their mutant characteristics. Stock cultures were prepared in nutrient broth with DMSO and forzen at -70°C.

The protocol used for the quantitative plate incorporation assays of the CPPE-1 powder coal-fired fly ash sample is outlined in Figure 1. Pour plastes were made by adding 0.1 ml of an overnight nutrient broth culture of the tester strain to 2 ml of molten top agar (45°C) in sterile 16 x 120 mm test tubes, followed by 0.1 ml of the chemical to be tested and 0.5 ml of the S-9 mix. Small amounts of histidine and biotin were added to the top agar to allow the bacteria to undergo several divisions, a necessity in many cases for mutagenesis. The tubes were mixed by rotating and poured onto the surface of a minimal agar plate. The plates were tilted quickly so the entire surface of the agar plate was covered with top agar. This procedure was performed in approximately 20 sec, since the enzyme activity in the S-9 mix can be inactivated rapidly. In addition to the test plates, a control plate for spontaneous reversion rate was made for each tester bacterial strain without the test chemical. All petri dishes were incubated at 37°C for 2 days, after which the number of revertant colonies was counted. As appropriate sterility checks were made on all mutagen solutions.

The final fly ash sample from an electrostatic precipitator (CPPC-1) was tested in powder form and as the superantant of a 50 µg/ml and 500 µg/ml suspension in organ culture medium (CMRL 1066), both in the presence and absence of the S-9 mix.

A test was considered positive if the number of mutant colonies in presence of the test compound was significantly greater than the number on control (spontaneous revertants) dishes. The significance was tested by Student's t test or over a series of experiments by an analysis of variance. From mutagenicity data provided by the Ames test[8], a compound was considered positive if it had at least double the number of mutants per plate over the sponteneous revertants. The results were expressed as the mean of duplicate observations.

TRACHEAL TRANSPLANTS

Methodology used for tracheal transplants and preparation of pellets for fly ash and BP (positive control) application were as follows:

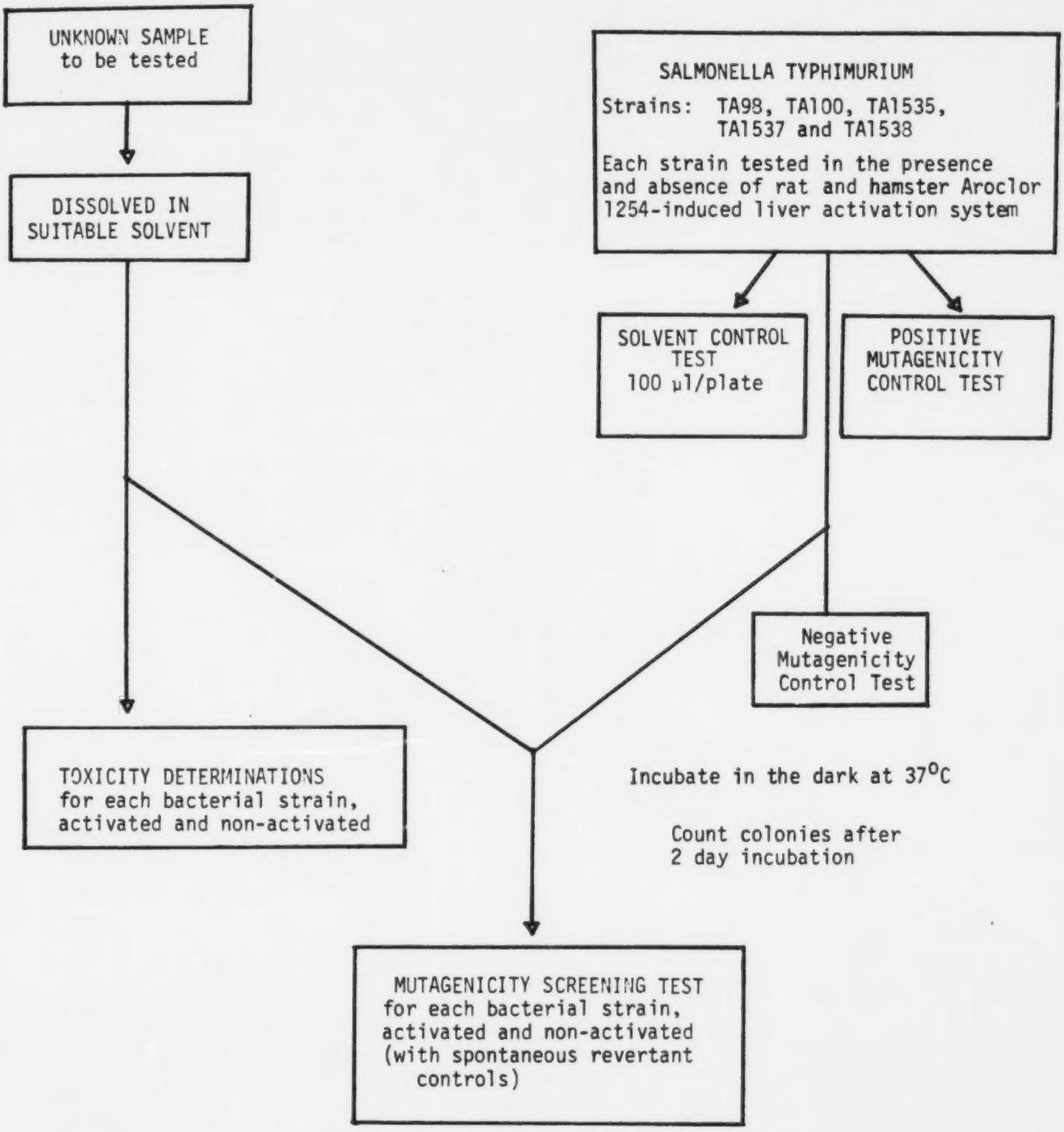

Figure 1: Procedure for Salmonella Mutagenicity Screening Test (Plate Assay)

Preparation of Beeswax Pellets

Beeswax was used as the vehicle to sustain release of the test compounds and positive carcinogen control. To prepare pellets, white beeswax was sterilized by heat filtration and mixed with cholesteryl laurate at a ratio of 1:9 (beeswax:cholesterol). The release rate of carcinogen in this mixture was approximately 1 µg/day for pellets containing 100 µg BP (Pal, Oak Ridge National Labs., personal communication). Weighed amounts of test compound and beeswax:cholesterol were melted in a scintillation vial at approximately 120°C and mixed while hot with a vortex mixer. An appropriate aliquot of the mixture was transferred to a preheated pellet mold (as designed by Griesemer, *et al.*, Oak Ridge National Labs.)[9] for preparation of pellets. After cooling, the pellets were expelled from the mold with a metal rod and stored under sterile conditions at room temperature.

Preparation of Whole Tracheas for Transplanting

Fischer 344 rats and B10 F1D Alexander hamsters used as tissue donors, were anesthetized by an ip injection of Brevital (sodium methohexital, Eli Lilly, Indianapolis, IN). The entire underside of the animal (pinned out on a cork board) was soaked with liquid paraffin to prevent hair from getting into the open area around trachea. Sterile conditions were used from this point in the procedure. The abdominal cavity was opened and dorsal aorta cut to eliminate blood around trachea area. A sterile 4 x 4 pad was used to absorb blood. The skin was incised in a longitudinal direction from the upper neck to the abdominal cavity. The skin then was pulled back flat and cuts made along each side of the rib cage and along the side of the trachea, exposing the trachea. A sterile paper towel was placed over the exposed area. Light pressure was applied causing blood to form on the towel. The bloody area on the towel was cut away, exposing the tracheal area only. The esophagus was separated from the trachea by inserting large scissors between the two organs, with care given to avoid cutting esophagus. Using a 18 gauge needle, the trachea was flushed with sterile Hanks' balanced salt solution with antibiotics. A small amount of Hanks' medium was used so that it didn't flood the nasal cavity and back into the trachea. A small incision was made at the larynx and pellet containing the test material was inserted into the trachea. A sterile polyethylene tube was placed next to the exposed trachea and sutured at both ends to the trachea. The trachea was cut below the sutures at the bifurcation and above the larynx. The extra tubing was cut so the trachea and tubing were the same length, and placed in a sterile petri dish containing L-15 medium.

Procedure for Tracheal Transplants and Test Compound Application

Recipient rats and hamsters were anesthetized by inhalation of methoxyflurane (Metofane, Pitman-Moore, Inc., Washington Crossing, NJ). The hair coat on the back was shaved distal to the scapula with electric clippers, chemically dipilated and sterilized. A 2 cm longitudinal incision was made on the dorsal midline between the shoulders. The skin was retracted

and subcutaneous pockets prepared on both sides (subscapular region) by blunt dissection. The donor trachea, with attached polyethylene tubing to prevent curling of the transplant, was inserted into the deep subcutaneous tissue (pocket). The incision was closed with metal clips (autoclips).

SECTION 5

RESULTS AND DISCUSSION

Initially, studies were conducted to determine:

- the concentration of hamster-adapted influenza A/PR-8 (HON1) virus that cause a minimum effect and the no-measurable effect in tracheal ring organ culture from adult hamsters and in whole tracheal organ cultures from 4 to 6-day old hamsters,
- the most efficient method for exposing tracheal organ cultures to particulate pollutants,
- the concentration of particulate pollutant coal-fired (fly ash) that cause a minimum effect as well as no-measurable effect in tracheal organ culture, as measured by alterations of ciliary activity and structural changes.

Results of these experiments formed the basis for the selection of appropriate concentration of the virus and the effluent to be used in the studies of interaction between the air pollutant and infectious agent.

EFFECT OF INFLUENZA A/PR-8 VIRUS CONCENTRATION ON TRACHEAL EPITHELIUM

Tracheal Ring Cultures

Experiments were designed to determine the concentration of influenza A/PR-8 virus which cause no-measurable effect and a minimal effect in tracheal ring organ cultures. The dose-response relationship of influenza virus and its effect on mucociliary epithelium were examined by using a series of inocula representing approximately 3-fold increments. Since our previous studies[10] showed that an inoculum of $10^{3.5}$HAD virus caused cessation of ciliary activity with epithelial cell damage, ring explants were infected with $10^{3.0}$, $10^{2.5}$, $10^{2.0}$, or $10^{1.5}$HAD$_{50}$ influenza virus. Cytotoxicity was expressed in terms of cilia beating frequency and cytopathological alterations.

The mean data from two experiments (total of 24 rings per virus concentration per observation) are presented in Table 1 and 2. The uninfected control trachea organ cultures maintained actively beating cilia with smooth luminal surface for more than 13 days. By day 9 until the end of the 13 day observation period, the controls showed some swollen round cells in various

TABLE 1

DOSE-RESPONSE EFFECT OF INFLUENZA A/PR-8 VIRUS, AS REFLECTED IN CILIA BEATING FREQUENCY, IN TRACHEAL RING CULTURES

Virus Concentration (HAD_{50}/0.2 ml)	Mean Cilia Beating Frequency, Time After Infection, Days								
	<1 hr	2	5	6	7	8	9	12	13
Explant Control	1300[a]	1276[a]	1278[a]	1277[a]	1260[a]	1278[a]	1257[a]	1197[a]	1180[a]
$10^{1.0}$	1294[a]	1263[a]	1264[a]	1268[a]	1248[a]	1244[a]	1249[a]	1208[a]	1191[a]
$10^{1.5}$	1305[a]	1281[a]	1258[a]	1261[a]	1260[a]	1250[a]	1249[a]	1201[a]	1171[a]
$10^{2.0}$	1299[a]	1280[a]	1236[ab]	1233[b]	1208[b]	1181[b]	1155[b]	1105[b]	1098[b]
$10^{2.5}$	1294[a]	1263[a]	1233[b]	1209[b]	1184[b]	1165[b]	1082[c]	1090[b]	1043[c]
$10^{3.0}$	1288[a]	1235[a]	1200[b]	1168[c]	1130[c]	1074[c]	1010[d]	985[c]	933[d]

Values with the same superscript within each column are not significantly different ($P \leq 0.05$; Ducan's New Multiple-Range Test)

Results are the mean from duplicate experiments (24 rings/virus concentration/observation).

The standard error ranges which were <4% were omitted from the table for clarity.

TABLE 2

DOSE-RESPONSE EFFECT OF INFLUENZA A/PR-8 VIRUS, AS REFLECTED AS PERCENTAGE OF NORMAL EPITHELIUM, IN TRACHEAL RING CULTURES

Virus Concentration (HAD_{50}/0.2 ml)	Mean Percent of Normal Epithelium* Time After Infection, Days								
	<1 hr	2	5	6	7	8	9	12	13
Explant Control	98^{a}	96^{a}	95^{a}	95^{a}	90^{a}	90^{a}	86^{a}	73^{a}	75^{a}
$10^{1.0}$	96^{a}	96^{a}	94^{a}	90^{a}	88^{a}	77^{a}	75^{a}	75^{a}	63^{a}
$10^{1.5}$	98^{a}	95^{a}	95^{a}	79^{a}	77^{a}	75^{a}	71^{b}	66^{a}	61^{a}
$10^{2.0}$	96^{a}	95^{a}	75^{b}	59^{c}	42^{b}	36^{bc}	21^{c}	21^{b}	10^{b}
$10^{2.5}$	92^{a}	82^{bc}	70^{b}	60^{c}	35^{bc}	23^{cd}	10^{c}	10^{bc}	5^{b}
$10^{3.0}$	95^{a}	72^{c}	53^{c}	40^{d}	26^{c}	10^{d}	10^{c}	5^{c}	5^{b}

*Normal epithelium is defined as a smooth luminal surface with beating cilia.

Results are the mean from duplicate experiments (24 rings/virus concentration/observation).

Values with the same superscripts within each column are not significantly different ($P \leq 0.05$; Chi Square Distribution Test).

The standard error ranges which were <12% were omitted from the table for clarity.

states of protrusion from the epithelium; these changes reflect the increase in cytopathological alterations. Tracheal cultures infected with $10^{2.0}$HAD virus first showed signs of cytopathological changes at day 5. Although disseminated foci of epithelial cell degeneration and necrosis were more frequent in explants infected with $10^{1.5}HAD_{50}$ virus, they were similar to the foci of cell degeneration found in noninfected control explants during *in vitro* maintenance. By the sixth day of a significant decrease in cilia beating frequency was observed in tracheal rings infected with $10^{2.0}HAD_{50}$ virus. Once reduction in the mean percent of normal epithelium was observed, decline was rapid. The decrease in ciliary activity continued in parallel with shedding of epithelium. Thus, the concentration of virus necessary to produce a minimal effect in tracheal ring organ cultures was $10^{2.0}HAD_{50}$, and the no-measurable effect level was $10^{1.5}HAD_{50}$. These concentrations were then used in determining the effects of air pollutants on influenza virus infections.

Whole Tracheal Cultures

Similar studies were conducted with influenza A/PR-8 virus in whole tracheal explants from 4- to 6-day-old hamsters. The dose-response relationship of influenza virus was examined by using a series of inocula representing approximately 3-fold dilutions. Whole tracheas (total of 9 explants per virus concentration per observation) were infected with $10^{2.5}$, $10^{2.0}$, $10^{1.5}$ or $10^{1.0}HAD_{50}$ influenza virus. Cytotoxicity was expressed in terms of cilia beating frequency.

The data from three experiments are presented in Table 3. Uninfected control explants showed some decrease in ciliary activity after 14 days; this mainly reflecting adjustment to culture conditions. Explants examined immediately after infection (<1 hr) showed a pattern of ciliary activity similar to that of the uninfected controls. Two days following infection, explants infected with $10^{2.5}$ and $10^{3.0}HAD_{50}$ virus showed a slight but significant decrease in ciliary activity. No significant decrease in activity was noted after four days of infection. After six days a significant decrease in ciliary activity was observed in explants infected with $10^{2.0}$, $10^{2.5}$ and $10^{3.0}HAD_{50}$ virus. In explants exposed to $10^{2.0}HAD_{50}$ virus, a slight increase was noted during the 10th day of observation but again decreased slightly after 14 days. From the 6th day until the end of the 14th day observation period, explants infected with $10^{2.5}HAD_{50}$ virus showed a slight decrease in activity. When an inoculum of $10^{3.0}HAD_{50}$ virus was used, total cessation occurred by the eighth day.

From these observations the concentration of virus necessary to produce a minimal effect in whole tracheal organ cultures was $10^{2.0}HAD_{50}$ virus, and the no-measurable effect level was $10^{1.5}HAD_{50}$. These observations were similar to those in the tracheal ring culture study.

TABLE 3

DOSE-RESPONSE EFFECT OF INFLUENZA A/PR-8 VIRUS, AS REFLECTED IN CILIA BEATING FREQUENCY, IN WHOLE TRACHEAL ORGAN CULTURE

Virus Concentration (HAD_{50}/0.2 ml)	Mean Cilia Beating Frequency, Time After Infection, Days						
	<1 hr	2	4	6	8	10	14
Explant Control	1273[a]	1244[a]	1224[a]	1187[a]	1188[a]	1163[a]	1143[a]
$10^{1.5}$	1208[a]	1227[ab]	1214[a]	1210[a]	1180[a]	1134[ab]	1096[a]
$10^{2.0}$	1264[a]	1222[ab]	1206[a]	963[b]	1008[b]	1081[b]	1010[b]
$10^{2.5}$	1236[a]	1172[b]	1159[a]	944[b]	837[c]	973[c]	837[c]
$10^{3.0}$	1213[a]	1167[b]	1163[a]	363[c]	0*	0*	0*

* Not included in statistical analysis

Values with the same superscript with each column are not significantly different ($P \leq 0.05$; Duncan's New Multiple-Range Test).

Results are the mean from triplicate experiments (9 tracheas/virus concentration/observation).

The standard error ranges which were <2% were omitted from the table for clarity.

EVALUATION OF *IN VITRO* PARTICULATE EXPOSURE METHODOLOGY

The conventional static incubation of tracheal organ cultures does not provide conditions appropriate for optimal exposure of the explants. Therefore, experiments were conducted to determine the most effective method for exposure of differentiated respiratory epithelium in culture to the particulate pollutants (Table 4). Groups of tracheal ring explants were incubated with 150 μg carbon (<2μ size)/ml in either 60 x 15 mm plastic petri dishes (on rocker platform or mixed with magnetic stirrer) or a standard 16 x 125 mm screw cap tissue culture tube (in roller apparatus). Groups 1-4 consisted of two to three explants per dish exposed to carbon particles on a rocker platform (Bellco Glass, Inc., Vineland, NJ). The culture dishes were placed in a controlled atmosphere chamber and gassed with 5% CO_2 and balanced air. The chamber was then placed on a rocker platform which rocked at 10 cycles per min causing the medium with particulates to flow continuously over the epithelial surface. In groups 5-8, three explants per dish were exposed to carbon particles by mixing with a magnetic stirrer. A 7 x 2 mm magnetic stirring bar rotating at minimum speed was used. In groups 9 and 10, each tracheal ring was added to culture tubes and placed in a roller drum (Bellco Glass, Inc., Vineland, NJ) rotating at 0.5 rpm. Control cultures from each mode of exposure received fresh medium without carbon particles. All cultures were incubated at 37°C.

The medium was observed after 0.5, 1, and 3 hr of exposure to determine whether the carbon particles remained in suspension. In addition, physical damage and toxic effects were assessed in the exposed tracheal organ cultures at the end of 1 and 3 hr.

The effects of the various exposure techniques over a 3-hr expsoure period are summarized in Table 5. Tracheal explants exposed to carbon particles on a rocker platform exhibited slight pathological alterations compared to the controls as well as a slight decrease in the number of cilia. Control explants exhibited a smooth luminal surface with no decline in the beating frequency of cilia. Explants exposed to carbon particles using a magnetic stirrer showed destruction to the tracheal epithelium during the first 0.5 hr of exposure. After 1 hr total destruction of the epithelium was observed. A similar destruction to the epithelium was observed in control cultures without carbon particles, indicating that the damage resulted from the magnetic stirring and not the carbon particles. Exposure to carbon in the roller tube system did not induce any striking cytotoxic effects on the epithelial surface during the first 0.5 hr of exposure. We noted that some of the carbon particles began settling out in the bottom of the culture tubes. Exposure for 1 hr produced a cytotoxic effect consisting of loss of normal columnar epithelial cells with moderate cell fragmentation. Beating cilia were observed in about half of the epithelium. However, accurate assessment was difficult since the cellular debris adhered to the lumen making ciliary activity reading difficult. During this 1 hr exposure, most of the carbon particles settled to the bottom of the culture tube. After 3-hr exposure, very few carbon particles remained in suspension. Portions of the epithelium were heavily coated with carbon particles. The epithelium showed a jagged appearance with occasional beating cilia. Control cultures maintained in normal medium showed few abnormalities, although slowing of ciliary activity and loss of 10-15% characteristic columnar alignment were detected.

TABLE 4

EXPERIMENTAL DESIGN TO EVALUATE
in vitro EXPOSURE METHODOLOGY

Group No.	Exposure Method	Exposure Period (hr)	Amount of Carbon/Dish (µg)	Amount of Medium/Dish (ml)	No. of Explants
1	Rocker Platform	1	300	2	8
2	Rocker Platform	1	450	3	8
3	Rocker Platform	3	300	2	8
4	Rocker Platform	3	450	3	8
5	Mixed-Magnetic Stirring	1	300	2	12
6	Mixed-Magnetic Stirring	1	450	3	12
7	Mixed-Magnetic Stirring	3	300	2	12
8	Mixed-Magnetic Stirring	3	450	3	12
9	Roller Tube	1	450	3	10
10	Roller Tube	3	450	3	10

TABLE 5

EFFECTIVENESS OF ROCKER, MAGNETIC STIRRING AND ROLLER EXPOSURE METHODS FOR EXPOSING <2μ CARBON PARTICLES TO HAMSTER TRACHEAL ORGAN CULTURE

Mode of Exposure	Time of Exposure (hr)	Observation and Effect
Rocker Platform	Control	Smooth luminal surface of epithelium with beating cilia. Slight decrease in number of cilia during 3 hr period.
	0.5	Carbon particles stayed in suspension in both 2 and 3 ml dishes. No physical damage or toxic effect.
	1	Carbon particles suspended as at 1/2 hr exposure period. No effect or damage to epithelium.
	3	Particulates in all dishes remained suspended. No Toxic effect or physical damage to luminal surface. Beating frequency of cilia declined from controls.
Magnetic Stirring	Control	Total destruction to epithelium after 1 hr exposure.
	0.5	Carbon particles in suspension. Epithelium appeared grossly degenerated. Cessation of ciliary activity.
	1	Total destruction to the tracheal epithelium in both 2 and 3 ml dishes.
Roller Tube	Control	Slowing of ciliary activity with slight pathological alterations to epithelium.
	0.5	Carbon particles sedimented on the epithelial surface. Most particles cleared by mucociliary action. Some carbon particles settled out in bottom of tube.
	1	Most carbon particles settled to bottom of tube. Cytotoxicity consisting of cell fragments and dying cells in lumen, vacuolated cells and ciliostasis in half of ciliated cells.
	3	Very few carbon particles in suspension. Portion of epithelium heavily coated with carbon particles. Portions of epithelium had jagged appearance with few beating cilia.

Inasmuch as the rocker platform procedure allowed the carbon particles to remain in suspension, produced only slight physical damage to the epithelium of the test and control cultures, and only a slight reduction in beat frequency, this methodology was employed in experiments using particulate pollutants.

EFFECT OF COAL-FIRED FLY ASH ON SALMONELLA TYPHIMURIUM MUTANTS

The CPPE-1 powder sample (EP-4) received from the EPA was tested for mutagenicity by the *Salmonella* mutagenicity test to implement the appropriate safety measures in the experimental procedures if necessary. The sample was tested in powder form and as the supernatant of a 50 µg/ml and 500 µg/ml suspension in tissue culture medium (CMRL 1066). Because of its use in our organ culture system, CMRL 1066 medium was chosen as the vehicle used to introduce the particulate sample and supernatants into the test system. The mutagenic activities of the three samples on five *S. typhimurium* bacterial strains under the test conditions specified are presented in Table 6. Both powder and supernatant samples possessed no detectable mutagenic activity under the test conditions.

EFFECT OF *IN VITRO* EXPOSURE TO FLY ASH ON HAMSTER TRACHEA IN ORGAN CULTURE

Tracheal Ring Culture

Cytotoxicity studies--Experiments were designed to determine the minimum-effect dose as well as the no-effect dose of the coal-fired fly ash sample (EP-4) from the final stage of an electrostatic precipitator. Tracheal ring organ cultures were exposed *in vitro* to 5, 10, 50, 100, 250, or 500 µg fly ash/ml for 1 hr/day, 4 days for the first week and 5 days for the second week using a rocker platform which rocked at 10 cycles/min causing the medium with particulates to flow continuously over the epithelial surface. Cytotoxicity resulting from exposure to fly ash was expressed as ciliary activity and cytopathology from a total of 24 observations of six tracheal rings from three hamsters.

At <1 hr and at any given 24 hr period, control and pollutant treatment means were compared using Duncan's New Multiple-Range Test. The effects of various concentrations of the fly ash sample on cilia beating frequency (Table 7) show that tracheas exposed to 50 µg/ml or less were not significantly different from the controls with a single exception at day 1. At 100 µg/ml the decline in cilia beating frequency was, in general, significantly different from the controls. Explants exposed to 250 and 500 µg/ml, showed a significantly greater decline in cilia beating frequency compared to controls. These concentrations also usually depressed ($P \leq 0.05$) beating frequency when compared to the four lower fly ash concentrations.

At 50 µg/ml or less, fly ash does not have a deleterious effect on the ciliated epithelium as measured by cilia beating frequency. Above this concentration, toxicity appears to be related to the length of exposure to fly ash. Reduction in ciliary activity does not appear to be directly proportional to concentration of fly ash used.

TABLE 6

MUTAGENIC ACTIVITY OF CPPE-1 SAMPLE

Sample	Response with Salmonella Tester Strains									
	TA-98		TA-100		TA-1535		TA-1537		TA-1538	
	NA	A	NA	A	NA	A	NA	A	NA	A
CONTROLS										
Spontaneous Revertants	15	25	160	185	16	10	10	13	28	29
Positive Controls Methyl Methane-sulfonate (non-activation)	10		295		27		13		24	
2-aminofluorene (activation)		1498		850		16		18		1549
TEST SAMPLES[a]										
Powder	-*	-	-	-	-	-	-	-	-	-
500 µg/ml supernatant	-	-	-	-	-	-	-	-	-	-
50 µg/ml supernatant	-	-	-	-	-	-	-	-	-	-

NA - non-activated A = activated

[a] The test sample was applied as a powder or as the supernatant of its suspension in medium CMRL 1066 at the given concentration.

-* No mutagenic response obtained

TABLE 7

MEANS OF CILIA BEATING FREQUENCY IN TRACHEAL RING CULTURES AT VARIOUS INTERVALS DURING NINE 1 HR/DAY *IN VITRO* EXPOSURES TO COAL-FIRED FLY ASH

Fly Ash Concentration (µg/ml)	Mean Cilia Beating Frequency								
	Days in Culture								
	<1 hr	1	2	3	6	7	8	9	10
0	1221^{a}	1221^{a}	1203^{a}	1199^{a}	1189^{a}	1200^{a}	1182^{a}	1163^{a}	1154^{a}
5	1222^{a}	1190^{ab}	1196^{a}	1170^{a}	1186^{a}	1172^{a}	1173^{a}	1164^{a}	1135^{a}
10	1191^{ab}	1192^{ab}	1185^{a}	1158^{a}	1158^{a}	1159^{a}	1149^{a}	1134^{a}	1120^{a}
50	1177^{ab}	1172^{bc}	1170^{ab}	1146^{ab}	1119^{a}	1135^{ab}	1145^{a}	1116^{a}	1121^{a}
100	1150^{b}	1136^{cd}	1115^{bc}	1081^{bc}	1121^{a}	1089^{b}	1014^{b}	918^{b}	844^{b}
250	1072^{c}	1132^{cd}	1123^{bc}	1015^{c}	1041^{b}	950^{c}	930^{c}	839^{c}	797^{c}
500	965^{d}	1106^{d}	1093^{c}	900^{d}	882^{c}	819^{d}	742^{d}	575^{d}	341^{d}

Values with the same superscripts within each column are not significantly different ($P \leq 0.05$; Duncan's New Multiple-Range Test).

Mean of 24 observations of six tracheal explants.

The standard error ranges which were <4% were omitted from the table for clarity.

The luminal surface of explants exposed to 5 and 10 µg fly ash/ml, exhibited the same characteristics as the controls (Table 8). In tracheal rings exposed to 50 µg fly ash/ml or greater, the luminal surface no longer retained its smooth contour. The epithelial surface had a jagged appearance due to exfoliating cells, with a moderate number of beating cilia. The results demonstrate that fly ash produces cytopathological alterations depending upon concentration levels and length of exposure. Concentrations of at least 100 µg fly ash/ml were required to produce significant changes in cilia beating frequency. In contrast, 50 µg/ml produced significant morphological changes in the luminal surface with moderate number of beating cilia. These results indicate that cilia beating frequency alone is not a sufficient indicator of pollutant toxicity and should be used in conjunction with luminal surface examination.

In another series of experiments, ring cultures were exposed *in vitro* to various concentrations of fly ash for 3 hr per day. Cytotoxic changes were determined in organ cultures of tracheal epithelium exposed *in vitro* to 10, 50, 100 and 250 µg fly ash/ml for 3 hr/day, 4 or 5 day/week for 9 days. Tracheal organ cultures showing strong ciliary activity 24 hr after culturing were retained for the experiment. Cytotoxicity resulting from exposure to this particulate pollutant was expressed as cilia beating frequency and cytopathology. Data from each observation results from six tracheal explants from three hamsters.

The cilia beating frequency measured after rings were exposed to the various concentrations of fly ash is shown in Table 9. At the 10 µg/ml level the decline in cilia beating frequency was significantly different from the controls about half the time. This variation could be attributed to technical observations. Tracheal organ cultures exposed to the 50, 100 and 250 µg/ml concentrations showed significant declines in cilia beating frequency throughout the experiment.

A significant amount of damage to the epithelium immediately after the initial exposure to 100 and 250 µg fly ash/ml was observed (Table 10). The 10 µg/ml concentration exhibited similar characteristics as the controls until day 10 (after eight pollutant exposures) when toxic alterations began to appear. Concentrations of 50 µg/ml or greater produced significant morphological changes in the luminal surface 2 days following the initial exposure and continued throughout the experiment.

Histological studies--The mucosa of tracheal specimens maintained for 4 days in medium without fly ash was similar to the tracheal epithelium of the intact hamster (Table 11). By the seventh day in culture, focal changes in the epithelium were noted, mainly loss of ciliated cells. Columnar cells showed a decrease in height. Local areas of basal cell hyperplasia developed in some control cultures and in one explant the mucosa became stratified.

TABLE 8

PERCENTAGE OF NORMAL EPITHELIAL LAYER IN TRACHEAL RING CULTURES AT VARIOUS INTERVALS DURING NINE 1 HR/DAY *IN VITRO* EXPOSURE TO COAL-FIRED FLY ASH

Fly Ash Concentration (µg/ml)	Mean Percent of Normal Epithelium* Days in Culture								
	<1 hr	1	2	3	6	7	8	9	10
0	95^{a}	92^{a}	92^{a}	87^{a}	88^{a}	82^{a}	82^{a}	79^{a}	76^{a}
5	88^{ab}	87^{a}	87^{ab}	86^{a}	86^{a}	84^{a}	79^{a}	74^{a}	74^{a}
10	84^{ab}	86^{a}	84^{ab}	83^{ab}	80^{a}	76^{ab}	76^{a}	68^{ab}	69^{a}
50	79^{bc}	79^{a}	73^{b}	71^{bc}	65^{b}	63^{bc}	57^{b}	56^{b}	52^{b}
100	63^{cd}	64^{b}	58^{c}	65^{c}	59^{bc}	54^{cd}	46^{bc}	38^{c}	26^{cd}
250	64^{d}	56^{b}	50^{c}	50^{d}	48^{cd}	43^{de}	37^{cd}	25^{cd}	15^{cd}
500	57^{d}	58^{b}	48^{c}	45^{d}	43^{d}	35^{e}	26^{d}	14^{d}	8^{d}

*Mean of six tracheal explants; normal epithelium is defined as a smooth luminal surface with beating cilia.

Values with the same superscripts within each column are not significantly different ($P \leq 0.05$; Chi Square Distribution Test).

The standard error ranges which were <5% were omitted from the table for clarity.

TABLE 9

MEAN CILIA BEATING FREQUENCY IN TRACHEAL RING CULTURES AT VARIOUS INTERVALS DURING NINE 3 HR *IN VITRO* EXPOSURE TO COAL-FIRED FLY ASH

Fly Ash Concentration (µg/ml)	Mean Cilia Beating Frequency, Day in Culture								
	<1 hr	2	3	4	7	8	9	10	11
0	1216^{a}	1212^{a}	1216^{a}	1207^{a}	1180^{a}	1187^{a}	1185^{a}	1172^{a}	1158^{a}
10	1176^{b}	1160^{b}	1181^{a}	1169^{b}	1150^{ab}	1146^{ab}	1148^{b}	1123^{b}	1140^{a}
50	1149^{b}	1139^{bc}	1132^{b}	1119^{c}	1122^{bc}	1121^{bc}	1128^{b}	1057^{c}	1035^{b}
100	1136^{b}	1124^{c}	1105^{b}	1109^{c}	1100^{c}	1100^{bc}	1115^{b}	1018^{c}	1024^{b}
250	1159^{b}	1140^{bc}	1107^{b}	1079^{c}	1089^{c}	1000^{d}	980^{c}	931^{d}	885^{c}

Values with the same superscripts within each column are not significantly different ($P \leq 0.05$; Duncan's New Multiple-Range Test).

Mean of 24 observations of six tracheal explants.

The standard error ranges which were <3% were omitted from the table for clarity.

TABLE 10

PERCENTAGE OF NORMAL EPITHELIAL LAYER OF TRACHEAL RING CULTURES AT VARIOUS INTERVALS DURING NINE 3 HR *IN VITRO* EXPOSURE TO COAL-FIRED FLY ASH

Fly Ash Concentration (µg/ml)	Mean Percent of Normal Epithelium* Day in Culture								
	<1 hr	2	3	4	7	8	9	10	11
0	93^{a}	93^{a}	92^{a}	92^{a}	86^{a}	87^{a}	82^{a}	82^{a}	78^{a}
10	91^{a}	85^{ab}	86^{a}	85^{a}	78^{a}	77^{a}	74^{a}	64^{b}	58^{b}
50	87^{a}	75^{bc}	68^{b}	69^{b}	54^{bc}	48^{b}	53^{b}	47^{c}	35^{c}
100	74^{b}	76^{bc}	73^{ab}	70^{b}	59^{b}	54^{b}	44^{b}	43^{cd}	36^{cd}
250	75^{b}	71^{c}	68^{b}	55^{c}	43^{c}	47^{b}	44^{b}	34^{d}	25^{d}

*Mean of six tracheal explants; normal epithelium is defined as a smooth luminal surface with beating cilia.

Values with the same superscripts within each column are not significantly different ($P \leq 0.05$; Chi Square Distribition Test).

The standard error ranges which were <8% were omitted from the table for clarity.

TABLE 11

EPITHELIAL CHANGES IN HAMSTER TRACHEAL RING ORGAN CULTURES AT VARIOUS INTERVALS DURING TEN 1- OR 3-HR IN VITRO EXPOSURE TO FLY ASH

No. of Exposures	Fly Ash Concentration (μg/ml)	No. of Observations with Epithelial Changes											
		Within Normal Limits[a]		Desquamation of Superficial Cells[b]		Focal Basal Cell Hyperplasia[c]		Diffuse Basal Cell Hyperplasia[d]		Stratified Metaplastic Epithelium[e]		Epidermoid Metaplasia[f]	
		I	II	I	II	I	II	I	II	I	II	I	II
1	0	4	4	0	0	0	0	0	0	0	0	0	0
	10	4	4	0	0	0	0	0	0	0	0	0	0
	50	3	2	0	2	1	0	0	0	0	0	0	0
	100	-	2	-	2	-	0	-	0	-	0	-	0
	500	3	-	0	-	1	-	0	-	0	-	0	-
2	0	4	4	0	0	0	0	0	0	0	0	0	0
	10	4	4	0	0	0	0	0	0	0	0	0	0
	50	4	3	0	1	0	0	0	0	0	0	0	0
	100	-	2	-	2	-	0	-	0	-	0	-	0
	500	3	-	0	-	1	-	0	-	0	-	0	-
4	0	4	4	0	0	0	0	0	0	0	0	0	0
	10	0	3	0	1	4	0	0	0	0	0	0	0
	50	2	2	0	2	2	0	0	0	0	0	0	0
	100	-	2	-	2	-	0	-	0	-	0	-	0
	500	0	-	3	-	1	-	0	-	0	-	0	-
7	0	3	2	1	0	0	2	0	0	0	0	0	0
	10	0	2	2	0	2	2	0	0	0	0	0	0
	50	1	0	1	0	2	0	0	3	0	1	0	0
	100	-	0	-	0	-	0	-	2	-	2	-	0
	500	0	-	0	-	2	-	0	-	2	-	0	-
8	0	0	2	0	1	0	3	0	0	0	0	0	0
	10	0	0	0	2	0	2	0	2	0	0	0	0
	50	0	0	0	0	0	0	0	4	0	2	0	0
	100	-	0	-	0	-	1	-	4	-	1	-	0
	500	0	-	0	-	0	-	0	-	0	-	0	-
10	0	2	3	1	0	1	2	0	1	0	0	0	0
	10	1	0	1	0	1	2	0	2	1	2	0	0
	50	1	0	0	0	0	0	1	0	3	6	0	0
	100	-	0	-	6*	-	0	-	0	-	0	-	0
	500	0	-	0	-	2	-	0	-	4	-	0	-

I = 1 hr in vitro exposure; II = 3 hr in vitro exposure.

[a] Normal limits defined as pseudostratified ciliated, columnar epithelium with a single layer of basal cells.

[b] Exfoliation of differentiated surface epithelial cells; over 50% of the epithelial surface.

[c] Small foci with 2-4 cell layers.

[d] Diffuse hyperplasia 2-4 cell layers or foci with more than 4 cell layers.

[e] Layered polygonal and cuboidal cells with flattened superficial cells.

[f] More than 10% of the normal epithelium is replaced by squamous-like cells.

* Tissue damage, unable to make evaluation.

- not evaluated

The types of changes that occurred in hamster tracheal organ cultures during exposure to fly ash were similar to those described in the *in vivo* study. In cultures exposed to 10 and 50 µg fly ash/ml for 1 hr/day, focal basal cell hyperplasia was observed after four exposures, and at the 500 µg/ml level superficial cells began to slough. After seven exposures, evidence of basal cell hyperplasia was noted at all concentrations and stratified metaplastic epithelium was observed in cultures exposed to 500 µg fly ash/ml. Stratification of the epithelium was seen after 10 exposures at all concentrations of fly ash tested.

Exposure to 10, 50 and 100 µg fly ash/ml for 3 hr/day produced changes in the tracheal epithelium in all treatment groups after four exposures. The changes became more pronounced and extensive with higher concentrations of fly ash and longer exposures. From the seventh to the tenth exposure, epithelial alterations included occasional areas of cellular necrosis, larger areas of basal cell hyperplasia and stratified epithelium.

SEM studies--Because the density of ciliated and nonciliated cells varies within regions of the trachea, it was necessary to identify the particular area being evaluated. Ciliated cells were most numerous in the regions overlying the ventral surface of the cartilaginous rings; these areas were used for evaluation. Cilia of the tracheal epithelium were of uniform length and density in control explants.

Immediately after exposure to 10 µg fly ash/ml, separate particles remained on the surface of the rings and occasionally attached to the cilia, whereas in the 50 and 100 µg/ml groups the fly ash formed occasional clumps on the surface. Changes in the tracheal epithelium after one 3-hr exposure were obvious at the 100 µg/ml level and were recognized as loss of ciliated and goblet cells. In the areas of injury some cells were elongated, whereas others were rounded with overlying cellular debris consisting of necrotic cells. After four 3-hr exposures at all fly ash concentrations, a reduction in ciliated cells was noted. Similar results were obtained after 1-hr exposures. After seven 3-hr exposures, nonciliated and mucus-secreting cells were degenerating and flattened areas with short microvilli were observed. Epithelial surface changes from the eighth through the tenth exposure were characterized by increased areas of flattened cells. In areas where stratified metaplastic foci were seen, mounds of cells were observed protruding from the flat surface. In these areas only occasional patchy cilia were seen, many of which were shortened. Exposure to 50 and 500 µg fly ash/ml for 1 hr/day produced stratified metaplastic lesions similar to those described above with the exception of small mounds protruding from the surface.

These results indicate that fly ash produces cytopathological alterations depending on concentrations and duration of exposure. Concentrations of 10 µg fly ash/ml for example, produced cytopathological effects after eight pollutant exposures and histopathological alterations after nine exposures. One of the most significant findings at this concentration was epidermoid metaplasia. In respiratory mucosa, metaplasia is a common response of the epithelium to mechanical, chemical and metabolic stimuli. Exposure to 50 µg fly ash/ml produced significant changes in cilia beating frequency after 1 day, and after 2 days of exposure cytopathological alterations were noted. Comparison of these results argues for consideration of all indexes in assessing toxicity to the tracheal epithelium.

Whole Tracheal Organ Culture

Cytotoxicity studies--Studies were conducted to determine the concentration of fly ash that causes no-measurable effect and a minimal effect in whole tracheal organ cultures from 4- to 6-day-old hamsters. The tracheal cultures were exposed *in vitro* to 5, 10, 50 and 100 µg fly ash/ml 1 hr/day, 4 days for the first week and 3 days the second week for a total of 7 days. A total of nine tracheal cultures per fly ash concentration were used. The cultures were observed daily, after pollutant exposure. Cytotoxicity was expressed in terms of cilia beating frequency only. The ciliary activity was measured by focusing on the mucosa through the explant as well as at the edge of the culture.

The effects of various concentrations of the fly ash sample on cilia beating frequency (Table 12) show that the 5 µg/ml was not significantly different from the controls. At 10 µg/ml the decline in beating frequency was significantly different from the controls only at the 3 and 9 day measurements. Cultures exposed to 50 µg fly ash/ml showed a significantly greater decline from the 7th day. At the 100 µg/ml level, a significant decline was observed at all observation points.

The patterns of damage to the ciliated epithelium, at 50 and 100 µg fly ash/ml, was characterized by loss of ciliated epithelial cells and a flattening of the nonciliated cells. In a few cases, loss of cilia could be observed along with sloughing of epithelial cells. No significant damage was observed at lower levels of the pollutant.

It appears from these observations that the concentration of fly ash necessary to produce a minimal effect after 1 hr/day exposures in whole tracheal organ cultures was 10 µg/ml. The no-measurable effect level was 5 µg/ml. When compared with the effects produced in tracheal ring cultures, it can be seen that whole tracheal cultures are more sensitive than ring cultures as indicated by the earlier onset of significant changes at the lower concentrations.

In a corresponding study, whole tracheal organ cultures were exposed *in vitro* to 5, 10 and 50 µg CPPE-1 fly ash particles/ml for 3 hr/day, 4 day/week for a total of 8 days. The concentrations of fly ash was expressed in terms of cilia beating frequency. As before, ciliary activity was observed by focusing on the mucosa through the explant.

Results summarized in Table 13 show that the ciliary activity at the concentration 5 µg/ml was significantly different from the controls only after 10 days in culture. The cultures exposed to 10 and 50 µg fly ash/ml showed significant declines in beating frequency after 7 exposures (day 9 in culture). The concentration of fly ash necessary to produce a minimal effect in whole tracheal explants appears to be 5 µg/ml. The no-measurable effect level was not attained in this group of experiments.

TABLE 12

MEAN CILIA BEATING FREQUENCY IN WHOLE TRACHEAL CULTURES AT VARIOUS INTERVALS DURING SEVEN 1-HR *IN VITRO* EXPOSURE TO COAL-FIRED FLY ASH

Fly Ash Concentration (µg/ml)	Mean Cilia Beating Frequency						
	Day in Culture						
	<1 hr	2	3	4	7	8	9
0	1234[a]	1239[a]	1250[a]	1203[a]	1209[a]	1177[a]	1187[a]
5	1239[a]	1238[a]	1223[ab]	1203[a]	1203[a]	1197[a]	1158[ab]
10	1212[ab]	1206[a]	1190[bc]	1181[ab]	1184[a]	1144[a]	1128[b]
50	1197[ab]	1183[ab]	1175[bc]	1173[ab]	1112[b]	1088[b]	1070[c]
100	1179[b]	1156[b]	1162[c]	1151[b]	1107[b]	1076[b]	1066[c]

Values with same superscripts within each column are not significantly different ($P \leq 0.05$; Duncan's New Multiple-Range Test).

Results are the mean of 36 observations of nine tracheal cultures.

The standard error ranges which were <3% were omitted from the table for clarity.

TABLE 13

MEAN CILIA BEATING FREQUENCY IN WHOLE TRACHEAL CULTURES AT VARIOUS INTERVALS DURING EIGHT 3-HR *IN VITRO* EXPOSURES TO COAL-FIRED FLY ASH

Fly Ash Concentration (μg/ml)	Mean Cilia Beating Frequency							
	Day in Culture							
	1	2	3	4	7	8	9	10
0	1252[a]	1228[a]	1233[a]	1215[a]	2002[a]	1193[a]	1179[a]	1185[a]
5	1227[a]	1227[a]	1230[a]	1200[a]	1185[a]	1180[a]	1134[a]	1130[a]
10	1224[a]	1190[a]	1196[a]	1185[a]	1179[a]	1164[a]	1127[b]	1117[b]
50	1202[a]	1183[a]	1180[a]	1178[a]	1170[a]	1150[a]	1110[b]	1085[b]

Values with same superscripts within each column are not significantly different ($P \leq 0.05$; Duncan's New Multiple-Range Test).

Each value represents the mean of six tracheal explants.

The standard error ranges which were <4% were omitted from the table for clarity.

Histological studies--At the time of explantation, the tracheal epithelium was a simple columnar type with a single layer of basal cells and only a few cilia (Fig. 2). Differentiation of the columnar epithelial cells was maintained throughout the 2-week culture period in control cultures[6].

In explants exposed to 5 µg fly ash/ml for 1 hr/day, no effects could be observed after three exposures, while at 10 µg/ml the epithelium was characterized by loss of individual cell identity, pale-staining nuclei and several layers of irregular basal cells (Fig. 3). Epithelium of explants treated with 50 µg/ml was predominantly hyperplastic with scattered areas of stratified metaplasia (Table 14). After seven exposures tracheal explants exposed to the lowest concentration (5 µg/ml) of fly ash exhibited a marked diffuse basal cell hyperplasia with an occasional area of stratified metaplastic epithelium. Fly ash at concentrations of 10 and 50 µg/ml produced a metaplastic epithelium with moderate amounts of keratinization (Fig. 4). After 10 exposures to 10 µg fly ash/ml, epidermoid metaplasia involved the majority of the explants, producing large quantities of keratin (Fig. 5). Fly ash was moderately cytotoxic at 50 µg/ml concentration.

In tracheal cultures exposed to fly ash for 3 hr/day, abnormal changes similar to those described following 1-hr exposures were observed. The only difference noted was an earlier onset of morphological changes at 10 and 50 µg fly ash/ml.

SEM Studies--The ventral epithelial surface of the trachea from suckling hamsters was characterized by numerous microvillous cells and occasional well-developed cilia (Fig. 6). The epithelium of untreated control cultures appeared to have an equal number of ciliated and microvillous cells after 2 weeks in culture.

Tracheal epithelium showed no differences between 1 and 3 hr daily fly ash exposures. The surface characteristics of explants treated with 5 µg fly ash/ml were comparable to those observed in control cultures throughout the experimental period. The response of tracheal epithelium exposed to 10 µg fly ash/ml was dramatic. After 3 exposures, the epithelium was composed of large flattened cells, with loss of microvilli separating the cell apices; focal elevated areas forming mound-like structures were often observed (Fig. 7). Broad metaplastic areas consisting mainly of microvillous cells with an occasional ciliated cell could be seen after 6 exposures (Fig. 8); keratin formation also occurred throughout the epithelial layer. After 10 exposures, epidermoid metaplasia with keratinization was prominent (Fig. 9). At a concentration of 50 µg/ml metaplastic changes similar to those observed at 10 µg fly ash/ml occurred after fewer exposures.

A sequence of complex events is observed in the hamster tracheal mucosa following a series of daily exposures to fly ash. Initially, changes were manifested as sloughing of superficial epithelium followed by proliferation of basal cells (basal cell hyperplasia). Subsequently, areas of stratified metaplastic epithelium appeared.

TABLE 14

EPITHELIAL CHANGES IN WHOLE HAMSTER TRACHEAL ORGAN CULTURE
AT VARIOUS INTERVALS DURING NINE 1 AND 3 HR *IN VITRO* EXPOSURES TO FLY ASH

No. of Exposures	Fly Ash Concentration (μg/ml)	Within Normal Limits[a]		Desquamation of Superficial Cells[b]		Focal Basal Cell Hyperplasia[c]		Diffuse Basal Cell Hyperplasia[d]		Stratified Metaplastic Epithelium[e]		Epidermoid Metaplasia[f]	
		I	II	I	II	I	II	I	II	I	II	I	II
1	0	4	4	0	0	0	0	0	0	0	0	0	0
	5	4	3	0	0	0	1	0	0	0	0	0	0
	10	4	3	0	1	0	0	0	0	0	0	0	0
	50	4	2	0	1	0	1	0	0	0	0	0	0
3	0	4	4	0	0	0	0	0	0	0	0	0	0
	5	3	1	1	0	0	2	0	1	0	0	0	0
	10	2	0	0	0	2	2	0	1	0	1	0	0
	50	0	0	0	0	1	0	2	2	1	1	0	1
7	0	4	3	0	1	0	0	0	0	0	0	0	0
	5	0	0	0	0	0	0	4	1	0	2	0	1
	10	0	0	0	0	0	0	0	0	1	0	3	3
	50	0	0	0	0	0	0	0	0	0	1	4	3
9	0	3	2	0	1	1	1	0	0	0	0	0	0
	5	0	0	0	0	0	0	1	2	2	1	1	1
	10	0	0	0	0	0	0	0	0	2	0	2	4
	50	0	0	3	1*	0	0	0	0	0	1	1	2

I = 1 hr *in vitro* exposure; II = 3 hr *in vitro* exposure.

[a]Normal limits defined as pseudostratified ciliated, columnal epithelium with a single layer of basal cells.

[b]Exfoliation of differentiated surface epithelial cells;over 50% of the epithelial surface.

[c]Small foci with 2-4 cell layers.

[d]Diffuse hyperplasia 2-4 cell layers or foci with more than 4 cell layers.

[e]Layered polygonal and cuboidal cells with flattened superficial cells.

[f]More than 10% of the normal epithelium is replaced by squamous-like cells.

*Tissue damage, unable to make evaluation.

Figure 2. Trachea from 4-day-old hamster at time of explantation. H&E stain. x250

Figure 3. Tracheal explant after three 1-hr exposures to 10 µg fly ash/ml. Loss of cellular identity and irregularity of basal cell shape and stainability are seen. H&E stain. x250

Figure 4. Tracheal explant after seven 1-hr exposures to 10 µg fly ash/ml. Epithelium exhibits keratinizing epidermoid metaplasia. H&E stain. x250

Figure 5. Tracheal explants after ten 1-hr exposures to 10 µg fly ash/ml showing cornifying epidermoid metaplasia with sloughing keratinizing surface cells. H&E stain. x125

3

5

2

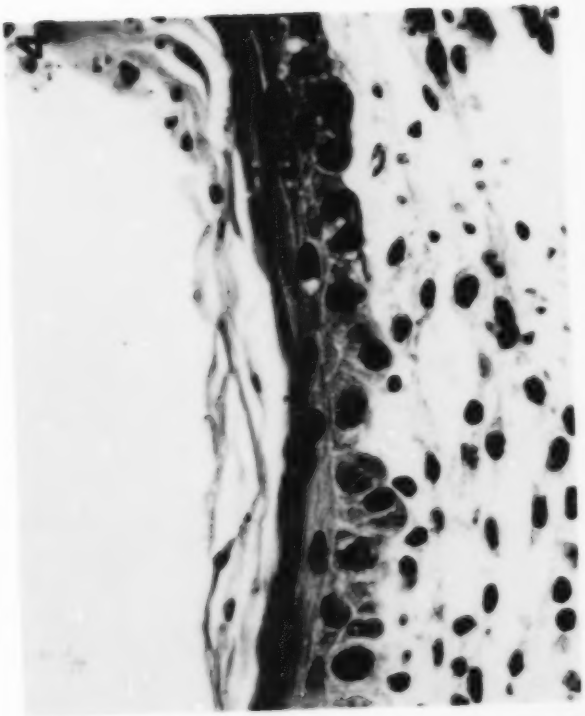
4

Figure 6. Ventral surface of trachea immediately after removal from 4-day-old hamster showing nonciliated microvillous cells with prominent outlines and occasional ciliated cells.
x1600

Figure 7. Surface of trachea from similar area as in Fig. 3 showing focal area of metaplasia forming mound-like structures.
x4000

Figure 8. Tracheal surface from similar area as in Fig. 4 showing large metaplastic cells surrounded by keratinizing surface cells.
x800

Figure 9. Surface of tracheal explant from similar areas as in Fig. 5 showing keratinizing surface cells sloughing from mucosal surface.
x4000

The change from basal cell hyperplasia to stratified metaplastic was one of the most significant findings in this study. Evidence indicates that the stratified epithelium arises from hyperplastic proliferation of basal and mucus cells and constitutes a prelude to epidermoid metaplasia[11]. In respiratory mucosa, metaplasia is a common response of the epithelium to mechanical, chemical and metabolic stimuli.

The results of these *in vitro* studies indicated that 10 µg fly ash/ml produced cytopathological changes after eight 3 hr/day exposures. Ciliary beat frequency at 10 µg/ml showed an inconsistant pattern of decline. The results presented in this report show histopathological alterations and change in surface topography after four exposures to 10 µg fly ash/ml. These results further indicate that cilia beating frequency and cytopathology alone are not adequate as pollution damage indicators and should be used in conjunction with light and scanning electron microscopy observations.

Comparative Toxicity of Fly Ash and Carbon

To determine whether the toxic effects to the tracheal ciliated epithelium was due to the chemical properties of the fly ash, tracheal ring organ cultures were exposed to 10, 100, 250 and 500 µg coal-fired fly ash or carbon (Sterling MTCT-6729, carbon black, 99.5% purity, Cabot Corp. Boston, MA)/ml for 3 hr/day, 5 days/week for 10 days using a rocker platform. The platform rocked at 10 cycles/min, causing the particles to stay suspended in the medium. A mean particle size of <2µ was used. At the end of each exposure period, six tracheal ring explants were evaluated for ciliary activity and cytopathology. Duplicate trials were performed on both fly ash and carbon samples.

A comparison of the effects of the various concentrations of fly ash and carbon (a relatively inert particle) on cilia beating frequency is shown in Table 15. The means for the 10 µg fly ash/ml did not differ significantly from similar concentrations of carbon. In general, tracheal cultures exposed to 100, 250 and 500 µg fly ash/ml showed a significantly greater decline in cilia beating frequency when compared to the same concentrations of carbon.

Immediately after exposure to 100 µg fly ash/ml, the ciliated epithelium showed significant cytological alterations when compared to that exposed to carbon particles (Table 16). Levels of fly ash from 100 µg/ml produced significant cytopathological alterations throughout the 11 day experimental period. The 250 µg/ml level of carbon produced significant cytopathological effects after eleven days of pollutant exposure.

This study shows that fly ash produced toxic effects at the 100 µg/ml level immediately after exposure while it requires a number of exposures at this concentration before a toxic effect with carbon was seen. Fly ash occurs in a variety of shapes and surfaces with many metals, polynuclear aromatic hydrocarbons and other organics present on the surface of fly ash[12]. These chemicals could be responsible for the toxicity to the epithelium which we observed.

Table 15

MEAN CILIA BEATING FREQUENCY IN RING CULTURES AT VARIOUS INTERVALS DURING NINE 3-HR *IN VITRO* EXPOSURES TO COAL-FIRED FLY ASH AND CARBON

Fly Ash & Carbon Concentration (µg/ml)	Mean Cilia Beating Frequency																	
	Day in Culture																	
	1		2		3		4		7		8		9		10		11	
	Carbon	Fly Ash	Carbon	Fly Ash	Carbon	Fly Ash	Carbon	Fly Ash	Carbon	Fly Ash	Carbon	Fly Ash	Carbon	Fly Ash	Carbon	Fly Ash	Carbon	Fly Ash
0 (Explant Control)	1236[a]	1221[a]	1234[a]	1221[a]	1203[a]	1231[a]	1219[a]	1199[a]	1191[a]	1189[a]	1188[a]	1200[a]	1182[a]	1161[a]	1178[a]	1163[a]	1182[a]	1154[a]
10	1203[a]	1191[a]	1221[a]	1192[a]	1198[a]	1185[a]	1174[a]	1158[a]	1153[a]	1158[a]	1158[a]	1159[a]	1131[a]	1149[a]	1154[a]	1134[a]	1177[a]	1120[a]
100	1184[a]	1150[a]	1203[a]	1136[b]	1194[a]	1115[b]	1156[a]	1081[b]	1138[a]	1121[a]	1166[a]	1089[b]	1115[a]	1014[b]	1132[a]	918[b]	1153[a]	844[b]
250	1104[a]	1072[a]	1184[a]	1132[b]	1173[a]	1123[b]	1130[a]	1015[b]	1162[a]	1041[b]	1156[a]	950[b]	1146[a]	930[b]	1122[a]	839[b]	1136[a]	797[b]
500	1178[a]	956[b]	1150[a]	1106[b]	1157[a]	1093[b]	1148[a]	900[b]	1147[a]	882[b]	1131[a]	819[b]	1042[a]	742[b]	1029[a]	575[b]	1119[a]	341[b]

Values with the same superscripts within each horizontal column at each interval are not significantly different ($P \leq 0.05$; Duncan's New Multiple-Range Test)

Results are the means from duplicate experiments 48 observations of 12 tracheal explants.

The standard error ranges which were <7% were omitted from the table for clarity.

Table 16

PERCENTAGE OF INTACT EPITHELIAL LAYER IN RING CULTURES AT VARIOUS INTERVALS DURING NINE 3-HR *IN VITRO* EXPOSURES TO COAL-FIRED FLY ASH & CARBON

Fly Ash & Carbon Concentration (µg/ml)	Mean Percent of Normal Epithelium* Day in Culture																	
	1		2		3		4		7		8		9		10		11	
	Carbon	Fly Ash	Carbon	Fly Ash	Carbon	Fly Ash	Carbon	Fly Ash	Carbon	Fly Ash	Carbon	Fly Ash	Carbon	Fly Ash	Carbon	Fly Ash	Carbon	Fly Ash
0 (Explant Control)	95[a]	95[a]	95[a]	92[a]	95[a]	92[a]	93[a]	87[a]	83[a]	88[a]	80[a]	82[a]	80[a]	82[a]	74[a]	79[a]	68[a]	76[a]
10	88[a]	84[a]	89[a]	86[a]	89[a]	84[a]	86[a]	83[a]	75[a]	80[a]	75[a]	76[a]	61[b]	76[a]	64[a]	68[a]	64[a]	69[a]
100	91[a]	63[b]	91[a]	64[b]	91[a]	58[b]	88[a]	65[b]	73[a]	59[b]	74[a]	54[b]	55[a]	46[a]	55[a]	38[b]	53[a]	26[b]
250	85[a]	64[b]	91[a]	56[b]	91[a]	50[b]	50[a]	90[b]	85[a]	48[b]	80[a]	43[b]	73[a]	37[b]	75[a]	25[b]	73[a]	15[b]
500	84[a]	57[b]	86[a]	58[b]	86[a]	48[b]	85[a]	45[b]	73[a]	43[b]	69[a]	35[b]	64[a]	26[b]	50[a]	14[b]	46[a]	8[b]

*Results are the means of 12 tracheal explants; normal epithelium is defined as a smooth luminal surface with beating cilia.

Values with the same superscripts within each horizontal column at each interval are not significantly different. ($P \leq 0.05$; Chi Square Distribution Test).

The standard error ranges which were <10% were omitted from the table for clarity.

EFFECT OF *IN VIVO* EXPOSURE TO FLY ASH ON HAMSTER TRACHEAL EPITHELIUM

In vivo studies in hamsters exposed to coal-fired fly ash were conducted to compare and validate the *in vitro* studies previously described.

Studies were conducted in which male hamsters were exposed to aerosols containing 1989 ± 76 μg/m^3 to the CPPE-1 (EP-4) particulate. The hamsters were exposed to the fly ash for 3 hr/day, 5 day/week for 2 weeks. The estimated hamster inhaled dose (HID) for this time period was 17 μg[13]. The deposition efficiency of this inhaled aerosol as calculated from Raabe, *et al*[14] was 2 μg. Immediately after, and at 1, 2, 3, 6, 7, 8, 9 and 10 days of exposure, the tracheas were removed and cut transversely into a series of 18 rings. The concentration of fly ash (2 mg/m^3) was chosen as the amount that might represent a maximum industrial environmental exposure. Alterations in cilia beating frequency and cytopathological changes were observed, and organ cultures were processed for histological and scanning electron microscopy. The experiments were performed in triplicate with a total of nine hamsters/group.

The data shown in Table 17 indicate that the mean cilia beating frequency for the 2 mg/m^3 treatment group was significantly lower than the filtered air group by the third day after exposure, and remained at a significantly lower level throughout the 10 day experimental period. Immediately after exposure to the fly ash, the tracheal mucosa showed significant cytological alterations which persisted throughout the 10 day experimental period. Immediately after exposure to the fly ash, the tracheal mucosa showed significant cytological alterations which persisted throughout the study at about the same level of damage when compared to the filtered air controls (Table 18).

Histological examinations showed the control hamsters to have normal tracheas, defined as having pseudostratified ciliated, columnar epithelium with a single layer of basal cells. Over the 2-week exposure period, an occasional control specimen showed epithelial degeneration of various degrees; occasional small foci of basal cell hyperplasia were seen, with some loss of cilia. Immediately after the first exposure to 2 mg fly ash/m^3 tracheas showed focal loss of cilia and focal areas of basal cell hyperplasia (Table 19). Of the nine specimens observed after two exposures to fly ash, three were normal, five showed diffuse basal cell hyperplasia and one specimen showed stratified metaplastic epithelium. An occasional mucous cyst could be observed. A similar pattern of epithelial change was observed after four exposures; although the number of specimens exhibiting stratification increased. The stratified metaplastic epithelium was characterized by layered polygonal and cuboidal cells. From the fifth through the ninth fly ash exposure, the tracheal epithelium was characterized by layered polygonal and cuboidal cells. From the fifth through the ninth fly ash exposure, the tracheal epithelium showed moderately large areas of basal cell hyperplasia and stratified epithelium. There were also areas of cellular necrosis with loss of cilia and desquamation.

TABLE 17

MEAN CILIA BEATING FREQUENCY IN HAMSTER TRACHEA AT VARIOUS INTERVALS DURING NINE 3 HR *IN VIVO* EXPOSURES TO COAL-FIRED FLY ASH

Fly Ash Concentration (mg/m^3)	Mean Cilia Beating Frequency, Number of Fly Ash Exposures								
	1	2	3	4	5	6	7	8	9
0 (Filtered Air Control)	1233	1224	1224	1227	1230	1230	1230	1238	1225
2	1210	1209	1180	1153*	1171*	1153*	1138*	1124*	1120*

*Significant difference from filtered air control cultures ($P \leq 0.05$; Duncan's New Multiple-Range Test).

Results are the means from triplicate experiments (36 observations of 9 tracheal rings).

The standard error ranges which were <2% were omitted from the table for clarity.

TABLE 18

PERCENTAGE OF NORMAL EPITHELIAL LAYER IN HAMSTER TRACHEA AT VARIOUS INTERVALS DURING NINE 3 HR *IN VIVO* EXPOSURES TO COAL-FIRED FLY ASH

Fly Ash Concentration (mg/m^3)	Mean Percent of Normal Epithelium† Number of Fly Ash Exposures								
	1	2	3	4	5	6	7	8	9
0 (Filtered Air Control)	95	88	85	86	85	88	86	90	88
2	78*	71	59*	63*	62*	59*	57*	60*	55*

†Results are the means of 9 tracheal rings; normal epithelium is defined as a smooth luminal surface with beating cilia.

*Significant difference from filtered air control cultures ($P \leq 0.05$; Chi Square Distribution Test).

The standard error ranges which were <4% were omitted from the table for clarity.

TABLE 19

HAMSTER TRACHEAL EPITHELIAL CHANGES AT INTERVALS AFTER IN VIVO EXPOSURE TO 1 OR 2 mg FLY ASH/m^3

No. of Exposures	Treatment	No. of Tracheal Rings with Epithelial Changes											
		Within Normal Limits[a]		Desquamation of Superficial Cells[b]		Focal Basal Cell Hyperplasia[c]		Diffuse Basal Cell Hyperplasia[d]		Stratified Metaplastic Epithelium[e]		Epidermoid Metaplasia[f]	
		I	II	I	II	I	II	I	II	I	II	I	II
1	Control	6	6	0	0	0	0	0	0	0	0	0	0
	Fly Ash	6	0	0	0	0	9	0	0	0	0	0	0
2	Control	6	6	0	0	0	0	0	0	0	0	0	0
	Fly Ash	6	3	0	0	0	0	0	5	0	1	0	0
4	Control	-	6	-	0	-	0	-	0	-	0	-	0
	Fly Ash	-	0	-	0	-	0	-	3	-	3	-	0
5	Control	6	3	0	0	0	3	0	0	0	0	0	0
	Fly Ash	5	0	0	0	1	4	0	0	0	2	0	0
6	Control	-	4	-	0	-	2	-	0	-	0	-	0
	Fly Ash	-	0	-	1	-	3	-	1	-	1	-	0
7	Control	6	3	0	0	0	3	0	0	0	0	0	0
	Fly Ash	3	0	0	0	3	0	0	4	0	2	0	0
8	Control	-	4	-	0	-	2	-	0	-	0	-	0
	Fly Ash	-	0	-	2	-	3	-	1	-	0	-	0
9	Control	4	3	0	0	2	3	0	0	0	0	0	0
	Fly Ash	0	0	0	2	2	0	2	4	2	3	0	0

I = 1 mg/m^3; II = 2 mg/m^3

[a] Normal limits defined as pseudostratified ciliated, columnar epithelium with a single layer of basal cells.

[b] Exfoliation of differentiated surface epithelial cells; over 50% of the epithelial surface.

[c] Small foci with 2-4 cell layers

[d] Diffuse hyperplasia 2-4 cell layers or foci with more than 4 cell layers.

[e] Layered polygonal and cuboidal cells with flattened superficial cells.

[f] More than 10% of the normal epithelial is replaced by squamous-like cells.

- not evaluated

The epithelial surface of tracheas from control hamsters consisted of three distinct cell types: ciliated, microvillous and goblet cells when examined by SEM. Areas of shortened cilia and many nonciliated cells with microvilli-covered surfaces were also observed. Following exposure to 2 mg fly ash/m^3, surface alterations consisted of decreased numbers of cilia and in some cases shortened cilia. After the second exposure there was an apparent increase in production of mucus. These changes continued through the fourth exposure. From the fifth through the seventh exposure, changes in the epithelial surface were more severe; not only did loss of cilia continue, but normal nonciliated cells were no longer present. In areas of stratified metaplastic epithelium the luminal surface area was increased due to flattening of the cells. Large flattened cells with either short microvilli or a smooth surface were seen.

Light microscopic and SEM examinations were also made on hamsters exposed to aerosols containing 1178 ± 37 (SE) $\mu g/m^3$ and compared with three exposed to 2 mg/m^3. Histological examinations of tracheal specimens after five exposures to 1 mg fly ash/m^3 showed epithelium similar to that obtained from control animals (Table 19). Observations made after seven exposures revealed focal areas of basal cell hyperplasia in one-half of the specimens. After nine exposures to 1 mg fly ash/m^3 none of the tracheas was normal. Stratified metaplastic epithelium, occasional diffuse basal cell hyperplasia, and focal basal cell hyperplasia were observed. In hamsters treated with 1 mg fly ash/m^3, changes in the ciliated surface as observed with SEM were less severe compared with animals exposed to 2 mg/m^3. Altered epithelium was usually limited to small focal areas during the first seven exposures. Following nine exposures, occasional large flat cells with short microvilli, typical of stratified metaplastic epithelium, could be seen.

RECOVERY FROM *IN VIVO* EXPOSURES TO FLY ASH

Male Syrian hamsters were exposed for 3 hr to 2055 ± 84 $\mu g/m^3$ of the CPPE-1 fly ash particle aerosol. The deposition efficiency of this inhaled aerosol was 2 μg[14]. After 2, 4, 5 and 9 exposures the tracheas from three hamsters were removed, cut transversely into a series of six rings and maintained as organ cultures. Ciliary activity and cytopathology in the explants were determined immediately after the final 3-hr exposure to the aerosol and after 1, 2, 3, 6 and 7 days maintenance in culture.

Table 20 shows the mean cilia beating frequency of ring cultures from hamsters following the various 3-hr fly ash exposures. The means after two and four exposures to fly ash did not differ significantly from the controls during the 7-day observation period. Tracheas maintained in organ culture following six and nine *in vivo* exposures to fly ash showed a significant decline in cilia beating frequency immediately after removal from a hamster and a significant depression of the mean beating frequency persisted through the 6th day.

TABLE 20

MEAN CILIA BEATING FREQUENCY OF TRACHEAL EPITHELIUM MAINTAINED *IN VITRO* AFTER ISOLATION FROM HAMSTERS EXPOSED *IN VIVO* FOR 3 HR/DAY TO 2055 µg FLY ASH/m^3

Number of 3 hr Exposures to 2055 µg Fly Ash/m^3	Mean Cilia Beating Frequency* Day After Final Exposure					
	<1 hr	1	2	3	6	7
0**	1259[a]	1250[a]	1255[a]	1238[a]	1214[a]	1263[a]
2	1212[a]	1194[a]	1196[a]	1202[a]	1209[a]	1203[a]
0	1234[a]	1220[a]	1221[a]	1215[a]	1219[a]	1211[a]
4	1206[a]	1200[a]	1197[a]	1203[a]	1192[a]	1189[a]
0	1270[a]	1254[a]	1228[a]	1262[a]	1233[a]	1214[a]
6	1131[b]	1126[b]	1172[b]	1167[b]	1170[b]	1177[a]
0	1230[a]	1230[a]	1219[a]	1212[a]	1208[a]	1205[a]
9	1089[b]	1120[b]	1137[b]	1135[b]	1154[b]	1174[a]

Values with the same superscript within each column of a treatment are not significantly different ($P \leq 0.05$; Duncan's New Multiple Range Test).

* Mean of 24 observations of 6 explants.

**Control hamsters held in exposure cages and exposed to filtered air for a corresponding number of 3 hr periods.

The standard error ranges which were <1% were omitted from the table for clarity.

The effects of the 3-hr exposures to fly ash on tracheal ciliated epithelium are shown in Table 21. Significant damage to the tracheal epithelium was seen immediately after 3-hr exposures to fly ash when compared to the filtered air controls. The same extent of cytological alterations was seen through the 2nd day following two or four 3-hr exposures, but the epithelium appeared similar to the controls by the 3rd day.

Immediately after six 3-hr exposures to the fly ash, the trachea showed significnat cytological alterations. The epithelial surface had a jagged appearance, as the result of exfoliating cells, with a moderate number of beating cilia. Recovery was noted on the 7th day of observation, after final exposure. The recovery of the damaged epithelium following nine exposures was similar to the tracheas of animals receiving six 3-hr exposures.

The earliest expression of cytotoxicity was alteration in the normal pattern of ciliated epithelium. Cilia beating frequency was not significantly altered until after six 3-hr exposures, while cytological alterations were seen after two exposures. It also appears that as the number of the 3-hr exposures to fly ash exposures increased, the longer it took for the ciliated epithelium to recover from the damage.

INTERACTIONS BETWEEN COAL-FIRED FLY ASH AND HAMSTER-ADAPTED INFLUENZA A/PR-8 VIRUS

In Vivo Experiments to Study the Interaction of Fly Ash and Influenza Virus

Influenza virus replication in hamster tracheal epithelium after aerosol infection--Four-week-old Syrian golden hamsters of the CrRGH(SYR) strain were infected with 100 HAD_{50}/0.1 ml influenza A/PR-8 virus by aerosol exposure. At <1 hr and 2, 3, 4, 7 and 10 days following infection, tracheas from two hamsters were homogenized, centrifuged and the supernatant frozen at -70°C and subsequently titered in Vero cells by the hemadsorption technique. The infectivity was calculated by the Reed and Muench method. The experiment was performed in duplicate with a total of 4 hamsters/group.

The HAD_{50} titers of progeny virus in homogenates of trachea at different times following aerosolization are shown in Table 22. No virus could be detected immediately after and 24 hr following infection. Viral replication was first demonstrated 2 days after infection and slowly increased, with a peak titer in tracheal homogenates being reached at 7 days following infection and declining slightly thereafter. These observations indicate that the respiratory epithelium of hamster trachea following aerosol infection is susceptible to influenza A/PR-8 virus infection.

TABLE 21

PERCENT OF NORMAL EPITHELIUM IN TRACHEAL RINGS MAINTAINED *IN VITRO* AFTER ISOLATION FROM HAMSTERS EXPOSED *IN VIVO* FOR 3 HR/DAY TO 2055 µg FLY ASH/m^3

Number of 3 hr Exposures to 2055 µg fly ash/m^3	Mean Percent of Normal Epithelium* Day After Final Exposure					
	<1 hr	1	2	3	6	7
0**	85[a]	90[a]	95[a]	85[a]	88[a]	87[a]
2	63[b]	68[b]	71[b]	80[a]	78[a]	82[a]
0	85[a]	90[a]	91[a]	90[a]	83[a]	83[a]
4	67[b]	65[b]	70[b]	80[a]	79[a]	71[a]
0	90[a]	88[a]	92[a]	96[a]	97[a]	95[a]
6	68[b]	63[b]	72[b]	77[b]	84[b]	83[a]
0	86[a]	88[a]	91[a]	90[a]	90[a]	88[a]
9	52[b]	51[b]	59[b]	68[b]	73[b]	78[a]

Values with the same superscript within each column of a treatment are not significantly different ($P \leq 0.05$; Chi-square Distribution Test)

* Mean of 6 explants; Normal epithelium = smooth luminal surface with beating cilia.

**Control hamsters held in exposure cages and exposed to filtered air for a corresponding number of 3 hr periods.

The standard error ranges which were <5% were omitted from the table for clarity.

TABLE 22

RATE OF INFLUENZA A/PR-8 VIRUS REPLICATION IN THE TRACHEA OF HAMSTERS AFTER INFECTION WITH 100 HAD_{50}/0.1 ml VIRUS AEROSOL

Days After Infection	Mean HAD_{50} Titers in Trachea Homogenates*	
	Expt. 1	Expt. 2
<1	0.0	0.0
1	0.0	0.0
2	1.8	2.2
3	3.0	ND**
4	1.8	2.7
7	3.4	4.0
10	2.5	3.3

* Titers expressed in $\log_{10}$ hemadsorption $dose_{50}$/0.1 ml
Results are the mean titers from four hamsters.

**Not done

Hamsters infected by aerosol exposure with virus and exposed immediately to fly ash--Four- to 6-week-old male Syrian hamsters were infected with 100 HAD_{50}/0.1 ml influenza A/PR-8 virus by aerosol exposure and immediately exposed to aerosols containing 1097 ± 29 $\mu g/m^3$ of the CPPE-1 fly ash particulate. The hamsters were exposed to the fly ash for 3 hr/day. The estimated HID for each 3-hr exposure was 9 µg and deposition efficiency was 1 µg. After 1, 2, 3, 6 and 10 exposures, tracheas from three hamsters were removed under sterile conditions and cut transversely into six rings (two/hamster). Control hamsters were held in exposure cages and exposed to filtered air for a corresponding number of 3-hr periods. Titration to determine viral replication was not performed in the *in vivo* experiments, because of the requirements of homogenates of the entire tracheal tissue.

The data shown in Table 23 indicates that the mean cilia beating frequency for the 1 mg fly ash/m^3 treatment group and virus-fly ash combination was significantly lower than the filtered air and virus groups three days after initial exposure. In addition to these two groups, the influenza virus-exposed group also showed a significant decline after 6 days. These three groups remained at equivalently significant lower levels compared to filtered air controls throughout the 11 day experimental period.

Immediately after a single exposure to the fly ash and virus-fly ash combination, the tracheal mucosa showed significant cytological alterations when compared to the filtered air control and virus exposed groups (Table 24). After 3, 6 and 10 exposures to ash alone, there was no significant difference from control. By the fourth and seventh days the pattern of effect of animals exposed to virus only and virus-fly ash combination was significantly different from the fly ash and filtered air. All except the virus-fly ash group returned to normal limits by day 10.

The tracheal epithelium of control animals has been described. Histological changes after the initial exposure to fly ash and virus-fly ash combination were characterized by a focal loss of ciliated cells and epithelial flattening. Observations after six exposures to virus-fly ash showed a diffuse basal cell hyperplasia, with many cells lacking apical morphological differentiation. By the eleventh day, 20 percent of the specimens showed extensive degeneration and cellular necrosis. In the remaining specimens, focal basal cell hyperplasia and an occasional area of stratified metaplastic epithelium could be seen.

Hamsters infected with virus aerosol and exposed 1 day later to fly ash--To determine if an ongoing infection is made more severe by exposure to a particulate air pollutant, hamsters were infected with virus by the aerosol route and exposed 1 day later to fly ash. After 1, 3, 4, 5 and 9 exposures (3 hr/day, 5 days/week), tracheas from three hamsters were removed and evaluated.

TABLE 23

MEAN CILIA BEATING FREQUENCY IN HAMSTERS INFECTED BY AEROSOLIZED VIRUS AND IN <1 HR EXPOSED TO FLY ASH FOR 3 HR DAILY, 5 DAYS/WEEK FOR UP TO 2 WEEKS

Concentrations		Mean Cilia Beating Frequency*				
		Number of Fly Ash Exposures				
Virus (HAD_{50}/0.1 ml)	Fly Ash (mg/m^3)	1	2	3	6	10
0	0	1226[a]	1226[a]	1226[a]	1236[a]	1236[a]
100	0	1232[a]	1220[a]	1210[a]	1185[b]	1175[b]
0	1	1225[a]	1199[a]	1185[b]	1179[b]	1180[b]
100	1	1226[a]	1219[a]	1182[b]	1177[b]	1169[b]

Values with the same superscript within each column of a treatment are not significantly different ($P \leq 0.05$; Duncan's New Multiple Range Test).

*Mean of 24 observations of 6 tracheal explants.

The standard error ranges which were <2% were omitted from the table for clarity.

TABLE 24

PERCENTAGE OF NORMAL EPITHELIAL LAYER IN HAMSTERS INFECTED BY AEROSOLIZED VIRUS AND IN <1 HR EXPOSED TO FLY ASH FOR 3-HR DAILY, 5 DAYS/WEEK FOR UP TO 2 WEEKS

Concentrations		Mean Percent of Normal Epithelium*				
		Number of Fly Ash Exposures				
Virus (HAD_{50}/0.1 ml)	Fly Ash (mg/m^3)	1	2	3	6	10
0	0	84^a	84^a	84^a	81^a	81^a
100	0	82^a	74^a	70^b	68^b	70^a
0	1	70^b	75^a	72^{ab}	78^{ab}	69^{ab}
100	1	61^b	72^a	61^b	59^b	63^b

* Mean of 6 tracheal explants; Normal epithelium is defined as a smooth luminal surface with beating cilia.

Values with the same superscript within each column of a treatment are not significantly different ($P \leq 0.05$; Chi-square Distribution Test).

The standard error ranges which were <5% were omitted from the table for clarity.

The effects of the various treatment groups and filtered air on the cilia beating frequency are shown in Table 25. The means of the virus-fly ash group for 1 and 3 days of exposure differed significantly from the virus, fly ash and control groups. After 4 days of exposure both fly ash and virus-fly ash treatments were statistically different from filtered air, but did not significantly differ from the virus group alone. After five exposures, a significant depression of the mean beating frequency was noted in all treatment groups and persisted throughout the remainder of the experiment.

Results summarized in Table 26 show that cytological alterations of the virus-fly ash group were significantly different from the filtered air controls, as well as the influenza virus or fly ash groups only, throughout the experiment. Animals exposed to fly ash were not different from control at any test time. In general, there were no significant morphological changes observed in the virus group. Histological examinations showed similar changes as described in the first group. The epithelium on a few rings showed focal areas of disordered histology and cellular atypia. These results correlate well with the cytological observations.

Hamsters exposed to fly ash, then infected immediately after final exposure to virus aerosol--The following studies were performed to determine the effect of the infection/pollutant exposure sequence. Hamsters were exposed for 1 week (five, 3-hr daily exposures) to aerosols containing approximately 1 mg fly ash/m^3 and infected immediately after the final exposure with influenza virus by the aerosol route. A total of six tracheal rings, two from each of three hamsters, were used for each observation. Parameters measured were the same described for the above experiments.

The effects of the different treatments on cilia beating frequency presented in Table 27 show that the virus-fly ash combination was the only group significantly different from the air control at the 3 and 5 day measurements. In the remaining observations of animals treated with fly ash, virus, and virus-fly ash combination (5 and 9 exposures), the decline in beating frequency was significant from air controls.

At 3 days, the luminal surface of tracheas from animals exposed to influenza virus or fly ash did not appear to show cytological alterations (Table 28), whereas the epithelial surface of the virus-fly ash group no longer retained the smooth contour. The surface had a jagged appearance due to exfoliating cells. A moderate number of beating cilia were observed. Similar toxic changes occurred in the virus, fly ash and virus-fly ash groups when observed 5 and 7 days following infection. Ten days following infection, this pattern of toxicity was observed only in the virus-fly ash group.

To extend these observations, hamsters were exposed for 2 weeks (five 3-hr daily exposures per week) to 1 mg fly ash/m^3, then infected with aerosolized influenza virus <1 hr after the final exposure. This experiment was performed with 3 hamsters/group (a total of 6 explants/observation).

TABLE 25

MEAN CILIA BEATING FREQUENCY IN HAMSTERS INFECTED BY AEROSOLIZED VIRUS AND 24 HR LATER EXPOSED TO FLY ASH FOR 3 HR DAILY, 5 DAYS/WEEK FOR UP TO 2 WEEKS

Concentrations		Mean Cilia Beating Frequency*				
		Number of Fly Ash Exposures				
Virus (HAD_{50}/0.1 ml)	Fly Ash (mg/m^3)	1	3	4	5	9
0	0	1226[a]	1226[a]	1226[a]	1236[a]	1236[a]
100	0	1237[a]	1220[a]	1210[ab]	1185[b]	1175[b]
0	1	1225[a]	1199[a]	1185[b]	1179[b]	1180[b]
100	1	1149[b]	1143[b]	1184[b]	1180[b]	1168[b]

Values with the same superscript within each column of a treatment are not significantly different ($P \leq 0.05$; Duncan's New Multiple Range Test).

*Mean of 24 observations of 6 tracheal explants.

The standard error ranges which were <1% were omitted from the table for clarity.

TABLE 26

PERCENTAGE OF NORMAL EPITHELIAL LAYER IN HAMSTERS INFECTED BY AEROSOLIZED VIRUS AND 24 HR LATER EXPOSED TO FLY ASH FOR 3 HR DAILY, 5 DAY/WEEK FOR 2 WEEKS

Concentrations		Mean Percent of Normal Epithelium*				
		Number of Fly Ash Exposures				
Virus (HAD_{50}/0.1 ml)	Fly Ash (mg/m^3)	1	3	4	5	9
0	0	84^{a}	84^{a}	84^{a}	81^{a}	81^{a}
100	0	82^{ab}	74^{a}	70^{b}	68^{a}	70^{a}
0	1	70^{a}	75^{a}	72^{ab}	78^{a}	69^{a}
100	1	41^{c}	43^{b}	45^{c}	55^{b}	40^{b}

*Mean of 6 tracheal explants; Normal epithelium is defined as a smooth luminal surface with beating cilia.

Values with the same superscript within each column of a treatment are not significantly different ($P \leq 0.05$; Chi Square Distribution Test).

The standard error ranges which were <6% were omitted from the table for clarity.

TABLE 27

MEAN CILIA BEATING FREQUENCY FOLLOWING FIVE 3-HR DAILY EXPOSURES OF HAMSTERS TO FLY ASH, THEN INFECTED WITH AEROSOLIZED INFLUENZA VIRUS <1 HR AFTER FINAL EXPOSURE

Concentrations		Mean Cilia Beating Frequency*			
		Days After Final Fly Ash Exposure			
Virus (HAD_{50}/0.1 ml)	Fly Ash (mg/m^3)	3	5	7	10
0	0	1226^{a}	1226^{a}	1236^{a}	1236^{a}
100	0	1230^{a}	1210^{ab}	1189^{b}	1175^{b}
0	1	1220^{a}	1190^{ab}	1188^{b}	1180^{b}
100	1	1184^{b}	1178^{b}	1175^{b}	1165^{b}

Values with the same superscript within each column of a treatment are not significantly different ($P \leq 0.05$; Duncan's New Multiple Range Test).

*Mean of 24 observations of six tracheal explants.

The standard error ranges which were <1% were omitted from the table for clarity.

TABLE 28

PERCENTAGE OF NORMAL EPITHELIAL LAYER FOLLOWING FIVE 3-HR DAILY EXPOSURES OF HAMSTERS TO FLY ASH, THEN INFECTED WITH AEROSOLIZED INFLUENZA VIRUS IMMEDIATELY AFTER FINAL EXPOSURE

Concentrations		Mean Percent of Normal Epithelium*			
		Days After Final Fly Ash Exposure			
Virus (HAD_{50}/0.1 ml)	Fly Ash (mg/m^3)	3	5	7	10
0	0	85^{a}	85^{a}	81^{a}	81^{a}
100	0	72^{ab}	70^{b}	66^{b}	68^{ab}
0	1	75^{ab}	70^{b}	65^{b}	70^{ab}
100	1	55^{b}	58^{b}	62^{b}	65^{b}

*Mean of six tracheal explants; normal epithelium is defined as a smooth luminal surface with beating cilia.

Values with the same superscript within each column of a treatment are not significantly different ($P \leq 0.05$; Chi-Square Distribution Test).

The standard error ranges which were <3% were omitted from the table for clarity.

Throughout the 10-day observation period, the pattern of ciliary activity, cytological alterations and histopathological changes were similar to those for the group exposed to fly ash for 1 week prior to viral infection (Tables 29 and 30). There was however a slightly lower beating frequency (10 to 15 beats/min) in the fly ash exposures at the 7 and 10 day observations, thereby reflecting significant differences.

In summary, the results from the four *in vivo* experiments indicate that hamsters exposed to a pollutant during an on-going infection (i.e., infected by aerosolized influenza A/PR-8 virus and 24 hr later exposed to 1 mg fly ash/m^3 for 3-hr/day, 5 days/week for 2 weeks) were most severely affected. The toxicity was mainly expressed as cytopathological and histopathological alterations along the luminal surface. The ciliary activity although significantly different from the ambient air control, showed only a slight decline in cilia beating frequency. In general, very little difference in ciliary activity was observed between the virus, fly ash and virus-fly ash groups.

The results of experiments in which hamsters were exposed to fly ash and then infected with influenza virus at various times after pollution exposure, indicate that fly ash inhalations had very little influence on subsequent influenza infection. A possibility for this lack of altered response is that fly ash exposures of the duration and concentration used were not sufficient to effect the virulence of subsequent infection. Also, little is known about effects of fly ash inhalation on other potential antiviral defense mechanisms such as a local IgA mediated immunity and interferon production.

In Vitro Coal-Fired Fly Ash Exposure of Tracheal Explants from Hamsters Infected *In Vivo* by Virus Aerosol

The purpose of this study was to determine the effects of *in vitro* exposure of coal-fired fly ash to tracheal explants from hamsters infected with aerosolized influenza virus and the correlation between the response in organ culture and the effect observed in hamsters after *in vivo* exposure. In the first series of experiments, tracheas from 4- to 6-week-old male hamsters infected with influenza virus aerosol produced from a suspension containing 100 HAD_{50}/0.1 ml, were removed immediately and processed as ring organ cultures. Groups of seven tracheal explants from four hamsters were then exposed to 5, 10 or 50 µg fly ash for 3 hr/day, 5 days/week for 10 days using a rocker platform (10 cycles/min), causing the medium with the fly ash to flow continuously over the epithelial surface. Cytotoxicity resulting from the infection and exposure to fly ash was expressed as ciliary activity, cytopathology and histopathology. Culture medium was collected at 1, 2, 3, 6 and 10 days after infection and prior to fly ash exposure, frozen at -70°C and subsequently titered to determine viral replication.

TABLE 29

MEAN CILIA BEATING FREQUENCY FOLLOWING TEN 3-HR DAILY EXPOSURES PER WEEK OF HAMSTERS TO FLY ASH, THEN INFECTED WITH AEROSOLIZED VIRUS <1 HR AFTER FINAL EXPOSURE

Concentrations		Mean Cilia Beating Frequency*			
		Days After Final Fly Ash Exposure			
Virus (HAD_{50}/0.1 ml)	Fly Ash (mg/m^3)	3	5	7	10
0	0	1226[a]	1226[a]	1236[a]	1236[a]
100	0	1225[a]	1200[a]	1175[bc]	1170[b]
0	1	1220[a]	1190[a]	1198[a]	1195[ab]
100	1	1163[b]	1165[b]	1156[c]	1161[b]

Values with the same superscript within each column of a treatment are not significantly different ($P \leq 0.05$; Duncan's New Multiple Range Test).

*Mean of 24 observations of six tracheal explants.

The standard error ranges which were <1% were omitted from the table for clarity.

TABLE 30

PERCENTAGE OF NORMAL EPITHELIAL LAYER FOLLOWING TEN 3-HR DAILY EXPOSURES PER WEEK OF HAMSTERS TO FLY ASH, THEN INFECTED WITH AEROSOLIZED VIRUS <1 HR AFTER FINAL EXPOSURE

Concentrations		Mean Percent of Normal Epithelium*			
		Days After Final Fly Ash Exposure			
Virus (HAD_{50}/0.1 ml)	Fly Ash (mg/m^3)	3	5	7	10
0	0	85^{a}	85^{a}	81^{a}	81^{a}
100	0	72^{ab}	70^{b}	66^{b}	68^{a}
0	1	75^{a}	70^{b}	65^{b}	70^{a}
100	1	60^{b}	60^{b}	62^{b}	73^{a}

*Mean of six tracheal explants; Normal epithelium is defined as a smooth luminal surface with beating cilia.

Values with the same superscript within each column of a treatment are not significantly different ($P \leq 0.05$; Chi-square Distribution Test).

The standard error ranges which were <2% were omitted from the table for clarity.

The effects of culture medium, fly ash and virus-fly ash exposure on cilia beating frequency are shown in Table 31. When the trachea were removed immediately after infection, the beating frequency in the virus-fly ash combination groups were generally significantly lower than the media and virus control groups throughout the 8-day exposure period, but did not show a significant decline when compared to the same fly ash concentration alone.

Cytopathological examinations of the tracheal mucosa showed similar alterations in the virus control group, 50 µg fly ash/ml group and virus-fly ash (10 and 50 µg/ml) combination group, 2 and 3 days after fly ash exposure (Table 32). After eight days, the percentage of normal epithelium in three virus-fly ash groups were significantly lower than the controls.

Histopathological examinations of tracheal mucosa from normal hamsters had a normal ciliated pseudostratified columnar epithelium during the first two days of observation and showed focal loss of cilia and basal cell hyperplasia thereafter. Explants from virus infected hamsters also showed focal areas of cilia loss and moderate desquamation of surface epithelium. In cultures exposed to 5, 10 and 50 µg fly ash/ml, focal basal cell hyperplasia was observed after 4 and 5 days while 8 days after the initial fly ash exposure a more diffuse hyperplasia was noted. Evidence of stratified metaplastic epithelium was also seen at this time in cultures exposed to 50 µg fly ash/ml. The epithelial surface from the virus-fly ash treated tracheal cultures showed focal areas of basal cell hyperplasia in two-thirds of the specimens examined throughout the 8 day observation period. Sloughing of superficial cells was also observed on day 8. Occasional diffuse basal cell hyperplasia was also seen. Moderate to marked ciliary loss was observed. These histopathological changes indicate that treatment with virus-fly ash combinations results in less severe damage than exposure to the fly ash alone.

The course of influenza virus replication in hamsters infected by aerosol and in <1 hr exposed *in vitro* to fly ash was similar for all three concentrations of fly ash (Fig. 10). The maximum titers were, however, higher in the explants exposed to 10 and 50 µg fly ash/ml. Maximum infectious virus titer in all treatment groups was seen 6 days following infection. In the virus infected control explants, maximum titer was not reached until the end of the experimental period (day 10).

Experiments were also performed in tracheas from hamsters infected with influenza virus aerosol produced from a suspension containing 100 HAD_{50}/0.1 ml and exposed *in vitro* 24 hr later to 5, 10 and 50 µg fly ash/ml, 3 hr/day, 5 days/week for 2 weeks. After selected intervals, seven tracheas were evaluated as previously described and culture medium collected and stored at -70°C until viral titration was performed.

The effects of exposure to 50 µg fly ash/ml alone on cilia beating frequency (Table 31) were significantly lower than those in the filtered air and virus control groups. Cultures infected with virus and exposed to 5 and 10 µg fly ash/ml showed no significant decline in beating frequency compared to filtered air and virus alone. Generally virus infected explants in combination with 50 µg fly ash/ml did show a significant decline from the

TABLE 31

EFFECT OF *IN VITRO* EXPOSURE TO COAL-FIRED FLY ASH ON MEAN CILIA BEATING FREQUENCY OF TRACHEAL EPITHELIUM FROM HAMSTERS INFECTED *IN VIVO* BY AEROSOLIZED VIRUS*

Dose of Aerosolized Virus (HAD_{50}/0.1 ml)	Fly Ash Concentration (µg/ml)	Mean Cilia Beating Frequency (beats/min)**								
		Trachea removed <1 hr after aerosolized virus infection					Trachea removed 24 hr after aerosolized virus infection			
		Days in Culture					Days in Culture			
		2	3	4	5	8	1	2	3	6
0	0	1249^{a}	1241^{a}	1214^{a}	1204^{a}	1207^{a}	1225^{a}	1233^{a}	1226^{a}	1222^{a}
100	0	1220^{a}	1210^{a}	-	1200^{a}	1175^{a}	1220^{a}	1220^{a}	1210^{a}	1195^{ab}
0	5	1200^{ab}	1188^{ab}	1182^{a}	1173^{ab}	1175^{ab}	1209^{ab}	1200^{ab}	1188^{ab}	1170^{bc}
0	10	1180^{b}	1181^{b}	1169^{ab}	1150^{b}	1146^{bc}	1176^{b}	1181^{b}	1169^{b}	1166^{bc}
0	50	1149^{b}	1139^{c}	1132^{b}	1119^{c}	1122^{cd}	1163^{b}	1149^{c}	1139^{c}	1124^{c}
100	5	1198^{ab}	1186^{ab}	1193^{a}	1163^{b}	1149^{bc}	1215^{a}	1212^{ab}	1214^{a}	1201^{a}
100	10	1171^{b}	1167^{b}	1171^{ab}	1158^{b}	1142^{bc}	1208^{ab}	1198^{ab}	1187^{ab}	1184^{ab}
100	50	1158^{b}	1147^{c}	1153^{b}	1142^{bc}	1097^{d}	1187^{ab}	1171^{b}	1160^{b}	1140^{c}

*Tracheal ring cultures were exposed *in vitro* to 5, 10 or 50 µg fly ash/ml 3 hr/day, 5 days/week for 2 weeks.

**Mean of 28 observations of seven tracheal explants.

Values with the same superscript within each column of a treatment are not significantly different ($P \leq 0.05$; Duncan's New Multiple Range Test).

The standard error ranges which were <2% were omitted from the table for clarity.

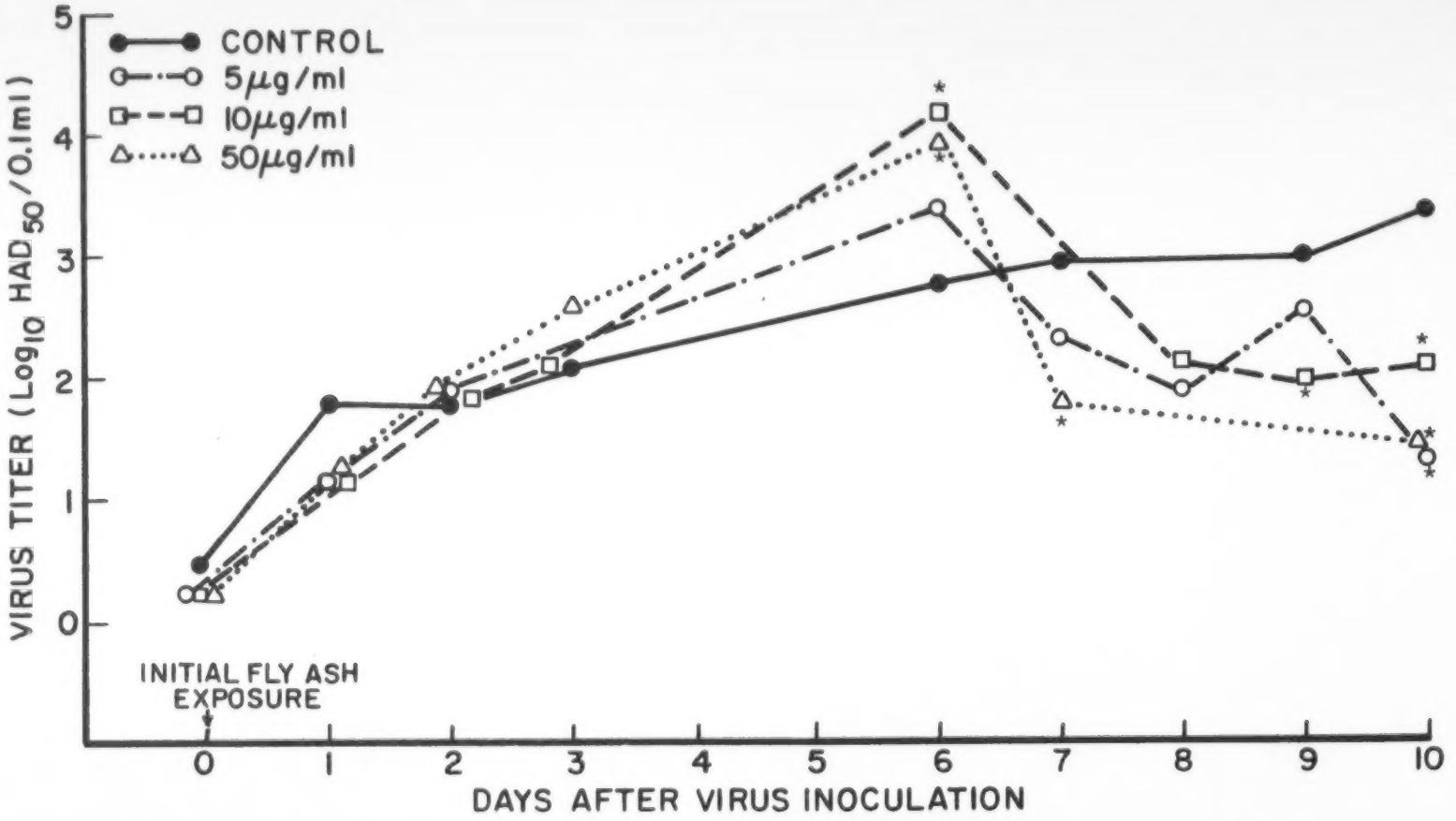

Figure 10. Rate of influenza A/PR-8 virus replication in tracheal explants infected *in vivo* by 100 HAD_{50}/0.1 ml aerosolized virus and in <1 hr exposed *in vitro* to fly ash for 3-hr daily, 5 days/week for 2 weeks. *P $\leq$ 0.05 as compared to virus control.

controls which, however, did not differ from the fly ash alone. Because of heavy epithelial outgrowth from the edges of the explants, ciliary activity observations could not be made after day 6.

The effects of the 3-hr exposures to fly ash 24 hr after infection with influenza virus aerosol are shown in Table 32. A significant damage to the tracheal epithelium was seen 1 day after exposure to the three virus-fly ash combinations when compared to the media control. The trachea epithelial surface had a jagged uneven appearance, as the result of exfoliating cells, with a moderate number of beating cilia distributed randomly along the mucosal surface. The same cytological alterations were seen in the 50 µg fly ash/ml treatment group two days after the fly ash exposure.

The mucosa of tracheal specimens from control hamsters exposed to filtered air only showed only a moderate degree of epithelial degeneration. This was mainly characterized by loss of cilia and focal basal cell hyperplasia. In tracheal cultures infected with virus only, moderate cilia loss was seen with some epithelial flattening resulting from desquamation of ciliated columnar and goblet cells. Changes that occurred in tracheal organ cultures exposed to varying concentrations of fly ash, described earlier, were also observed in the present study. In cultures exposed to 5 µg fly ash/ml, focal basal cell hyperplasia was observed after five exposures, progressing to a more diffuse basal cell hyperplasia after ten exposures. The epithelium of cultures exposed to 10 and 50 µg fly ash/ml for 10 days showed diffuse basal cell hyperplasia with occasional areas of necrosis and stratified metaplastic epithelium. These observations were more pronounced in the group exposed to 50 µg/ml. Exposure to virus infected tracheal mucosa to fly ash caused a series of changes that were, in general, time-related. After 3 days of daily exposure to 5 and 10 µg fly ash/ml, focal basal cell hyperplasia was observed with a moderate loss of cilia from the epithelial surface. The 50 µg fly ash/ml exposure resulted in some vacuolization of the epithelium in addition to the above changes. With further exposure to all three fly ash concentrations there were more diffuse basal cell hyperplasia with some cellular degeneration and vacuolation. Stratified epithelium was observed in only a few areas of the epithelium after day 10 in the 50 µg/ml exposure group. Deposits of fly ash were also seen between the mucosa and submucosa by day 3. Often, epithelial outgrowth was observed around the outside of the explants in the virus-fly ash treatment groups. When all parameters are considered, changes resulting from the virus-fly ash combination were more severe than in cultures exposed to fly ash alone.

The release of progeny virus from tracheal explants from hamsters infected *in vivo* and exposed *in vitro* 24 hr later to fly ash was followed by titration of culture medium sampled at intervals after infection. The titers were compared with those obtained with infected culture medium from explants exposed to fly ash immediately after infection. Growth curves are shown in Figure 11. Two days after infection the virus titer in medium from explants exposed to 10 µg fly ash/ml was approximately 15-fold higher than the 5 or 50 µg fly ash/ml group. Once the peak titer in fluid from the 10 µg fly ash/ml was reached it decreased only slightly thereafter. The titers of

TABLE 32

EFFECTS OF *IN VITRO* EXPOSURE ON TRACHEAL EPITHELIAL MUCOSA FROM HAMSTERS INFECTED *IN VIVO* BY AEROSOLIZED VIRUS*

Dose of Aerosolized Virus (HAD_{50}/0.1 ml)	Fly Ash Concentration (µg/ml)	Mean Percent of Normal Epithelium**								
		Trachea removed <1 hr after aerosolized virus infection					Trachea removed 24 hr after aerosolized virus infection			
		Days in Culture					Days in Culture			
		2	3	4	5	8	1	2	3	6
0	0	93^{a}	89^{a}	80^{a}	80^{a}	82^{a}	92^{a}	93^{a}	89^{a}	82^{a}
100	0	74^{bc}	70^{b}	66^{a}	61^{bc}	68^{b}	82^{ab}	74^{bc}	70^{b}	68^{bc}
0	5	92^{a}	88^{a}	85^{a}	81^{a}	77^{ab}	94^{a}	92^{a}	88^{a}	80^{a}
0	10	85^{ab}	86^{a}	85^{a}	78^{a}	75^{ab}	91^{a}	85^{ab}	86^{a}	76^{ab}
0	50	75^{bc}	68^{b}	69^{a}	62^{bc}	48^{c}	87^{a}	75^{bc}	68^{b}	54^{c}
100	5	86^{ab}	79^{ab}	80^{a}	70^{ab}	27^{d}	73^{bc}	71^{bc}	70^{b}	63^{bc}
100	10	71^{bc}	74^{ab}	68^{a}	50^{cd}	23^{d}	72^{bc}	68^{c}	66^{b}	62^{bc}
100	50	64^{c}	66^{b}	60^{b}	46^{d}	16^{d}	66^{c}	63^{c}	61^{b}	46^{c}

*Tracheal ring cultures were exposed *in vitro* to 5, 10 or 50 µg fly ash/ml 3 hr/day, 5 days/week for 2 weeks.

**Mean of seven tracheal explants; Normal epithelium is defined as a smooth surface with beating cilia.

Values with the same superscript within each column of a treatment are not significantly different ($P \leq 0.05$; Chi-Square Distribution Test).

The standard error ranges which are <7% were omitted from the table for clarity.

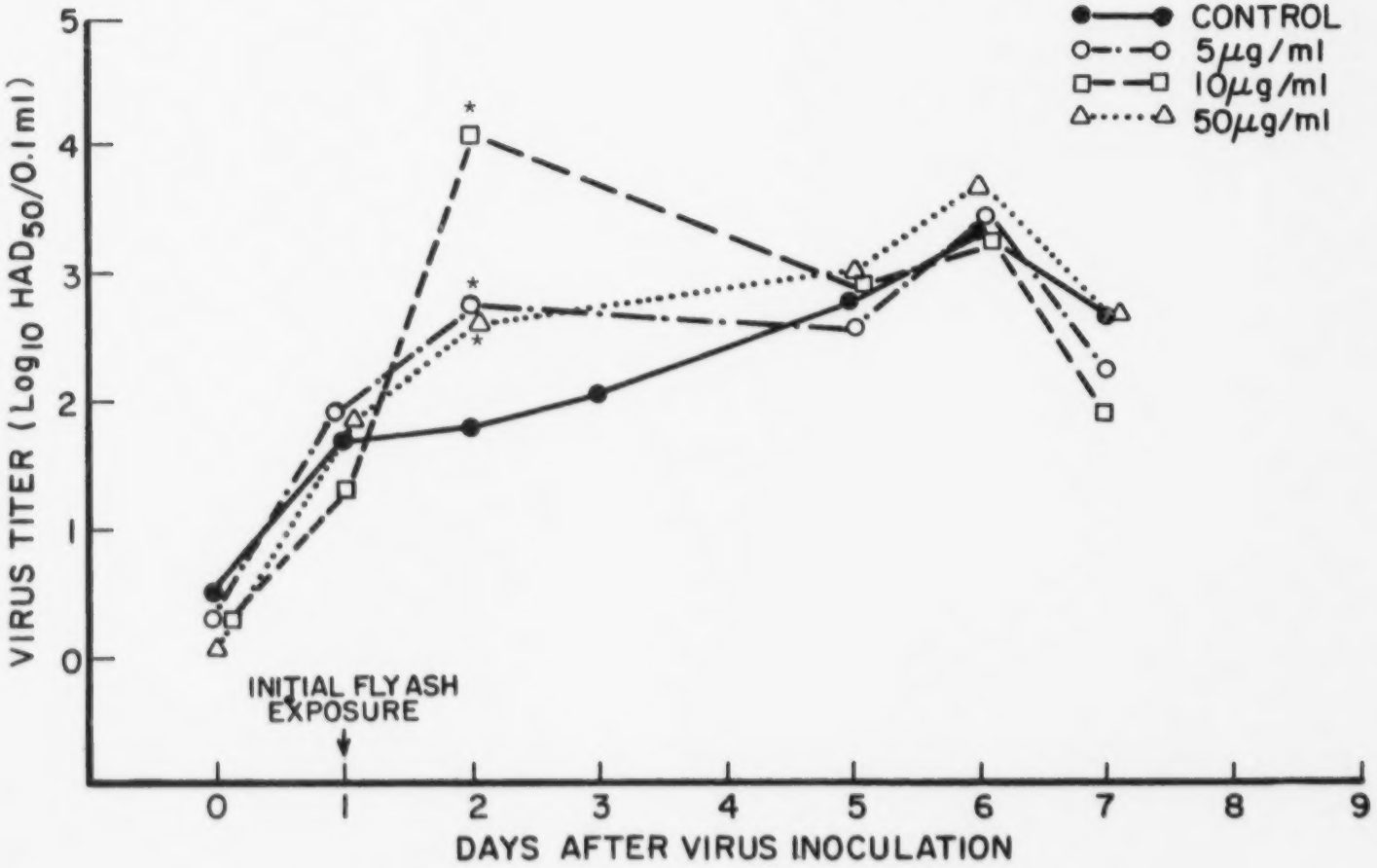

Figure 11. Rate of influenza A/PR-8 virus replication in tracheal explants infected *in vivo* by 100 HAD_{50}/0.1 ml aerosolized virus and 24 hr later exposed *in vitro* to fly ash for 3- hr daily, 5 days/week for 2 weeks. *P $\leq$ 0.05 as compared to virus control.

the 5 and 50 µg fly ash/ml treatment group did not peak until day 6. With the exception of the 10 µg fly ash/ml groups, titers obtained in both experiments (Figures 10 and 11) followed approximately the same pattern. No explanation can be given to explain the elevated viral titer in the 10 µg/ml group.

Tracheal cultures from hamsters infected with influenza virus aerosol and then exposed *in vitro* to fly ash showed morphologic alterations that were similar to those observed in the *in vivo* experiments. In comparison to *in vitro* exposures to fly ash alone, histological changes were less severe. Initial changes were characterized by focal loss of cilia and sloughing of superficial epithelium. This progressed to a diffuse basal cell hyperplasia with some degeneration and cellular necrosis. Moderate focal epithelial vacuolation was observed. Only occasional areas of stratified metaplastic epithelium appeared indicating the virus appeared to retard metaplastic changes. These changes were the same whether exposed immediately after infection or 24 hr post-infection. A similar decline in cilia beating frequency was observed with fly ash alone or virus-fly ash combination.

The results of these studies demonstrate that in hamsters infected with influenza virus 24 hr before exposure to 1 mg fly ash/m^3, the severity of the effects on the trachea epithelium increased. Experiments in which male CD_1 mice were exposed to 2 mg/m^3 of fly ash (CPPE-1) aerosol showed no significant changes when infected with influenza virus after 20 exposures to the particulate aerosol. However, a significant increase in mortality in male mice was seen when virus challenge immediately preceded five 3-hr particle exposures. In female mice, marked but not significant mortality increases were found under the same exposure conditions (Catherine Aranyi, personal communication). Previous studies in our laboratory with other air pollutants have shown that monkeys infected with influenza virus 24 hr before exposure to 10 ppm NO_2 died within 2 to 3 days after infectious challenge[15]. Cigarette smoke was shown to have similar effects on the resistance of mice to influenza infection[16].

Natusch and Wallace[17] have shown that particulate air pollutants can be lodged in respiratory tissue for extended periods. Coal-fired fly ash particulates, such as that used in our studies, once in the tracheal tissue may be absorbed and stored in the mucosa. Having localized in this respiratory tissue, these particles could participate in chemical reactions resulting in damage to the epithelium or decrease resistance to viral invasion by physical injury to the epithelial cells and by changing the production of surface secretions (mucous).

ESTABLISHMENT OF TRACHEAL GRAFTS

These initial studies were performed to determine whether tracheas from young adult rats and hamsters could be grafted without evidence of histocompatibility. Fischer 344 rats were used at the beginning of our experiments since it was the only isogenic host we had available. We have now initiated grafts of tracheas using the carcinogen-susceptible, B10 F_1D Alexander hamster, developed by Bio-Research Consultants, Inc. This inbred strain of hamster is isogenic.

Grafts that were maintained for 4-5 weeks showed well established vascularization and the lumen slightly dilated from accumulation of mucus. Host animals were given an ip injection of 0.5 μCi/gm of body weight of ^{3}H-methyl thymidine (sp. act. 20 Ci/mmole) 4 hr before removal of the graft. Tracheal epithelium from the grafts show histologically intact mucociliary epithelium (high columnar), submucosa and cartilage. In host hamsters, the labeling index varied from 0.01 to 0.1, i.e., number of labeled cells per number of cells counted. The labeling index of the tracheal grafts increased from 0.1 to 1.0.

Experiments were initiated to study the induction of preneoplastic and neoplastic lesions in grafted B10 F_1D Alexander hamster tracheas continuously exposed to CPPE-1 coal-fired fly ash. Four months after instillation of the fly ash-beeswax pellet, no tumors have appeared.

EVALUATION OF ADDITIONAL PARTICULATE POLLUTANTS IN TRACHEAL ORGAN CULTURE

The hamster tracheal organ culture bioassay system was used to provide additional health effects data on selected particualte materials. The pollutants included oil-fired fly ash, diesel fuel exhaust extract and cigarette smoke condensate. These air pollutants contain a variety of chemical carcinogens, the most commonly recognized being BP. The effect of this known carcinogen is described and compared with the responsiveness of hamster tracheal organ culture to the various pollutants in an effort to establish the relative potency of these substances.

Benzo(a)pyrene-Induced Changes in Tracheal Epithelium in Organ Culture

In order for an organ culture system to serve as a bioassay for chemical carcinogens it should be capable of exhibiting both cytotoxic and carcinogenic effects following exposure to these agents. Our protocol takes into account both types of effects. For our positive control, BP a known *in vivo* carcinogen was used.

Ring explants were exposed to 1, 3 or 10 μg BP/ml of culture medium for 3 hr/day, 5 days/week for 2 weeks, the same exposure period used for test compounds. BP was dissolved in acetone in a concentration not exceeding 0.1%. Ciliary activity and cytopathology were determined on four cultures/observation and four explants fixed and sections examined by light microscopy.

Cultures maintained during the 2 week exposure period in medium free of BP showed flattening of the epithelium but still maintained their pseudostratification with active beating cilia (range 1235 to 1189 bpm).

Explants exposed to 1 and 3 μg BP/ml showed evidence of epithelial changes. These cultures showed early flattening of ciliated and mucous cells, progressing to a stratified metaplastic epithelium with an occasional noncornifying epidermoid metaplasia. These changes were reflected as a decrease in normal cytopathology and ciliary beating frequency. Cultures exposed to 10 μg BP/ml showed early stratified hyperplastic epithelium. These cultures were atrophic after 11 exposures.

Cultures incubated in medium containing 0.1% acetone showed similar changes as 1 and 3 µg BP/ml after 7 and 11 exposures. As a result, BP solubilized in a solution with acetone did not appear to be appropriate for use as a control in our model. DMSO was therefore used as the solvent. At concentrations no greater than 0.01%, DMSO was found to be nontoxic when compared to the untreated controls. In explants exposed to BP dissolved in DMSO, similar results were obtained with 1 and 3 µg BP/ml in acetone as previously described. The cultures however, did not appear atrophic after 11 exposures.

Effects of Oil-Fired Fly Ash on Hamster Ciliated Tracheal Epithelium in Organ Culture

Tracheal ring organ cultures were exposed *in vitro* to 10, 50, 100 and 250 µg fly ash/ml, 3 hr/day, 5 days/week for a total of 10 days. Four cultures were observed daily and measurements of cilia beating frequency and cytopathology were made. An additional four explants were fixed for histological examination.

The results shown in Table 33 indicate that ciliary activity in explants exposed to all concentrations of fly ash were significantly reduced from the untreated control cultures. At the 250 µg fly ash/ml level, ciliostasis was observed 3 days after initial exposure. Cultures exposed to fly ash at 50 and 100 µg/ml produced ciliostasis on day 8. With a single exception at day 3, the beating frequency in the 10 µg fly ash/ml treatment group was significantly different from control cultures.

Cytopathological examinations of the tracheal mucosa showed significant alterations in the 10 µg fly ash/ml cultures 3 days after initiation of exposures and the ring cultures remained altered throughout the experiment (Table 34). Cultures treated with 50, 100 and 250 µg fly ash/ml showed significant differences at all exposure periods.

The results reported here demonstrated that tracheal epithelium from young adult hamsters exposed to oil-fired fly ash respond in a different manner than the fossil fuel (coal) fly ash previously reported. The response elicited by the 50, 100 and 250 µg oil-fired fly ash/ml doses was cytotoxic as indicated by ciliostasis and the absence of normal appearing epithelium. Similar concentrations of fly ash from coal fuel combustion showed a significant reduction in cilia beating frequency, but complete loss of ciliary movement was not observed. The degree of cytopathic change (during the early exposure periods) was also less severe in the coal fueled fly ash.

The tracheal epithelium of explants exposed to oil-fired fly ash exhibited pathological alterations in direct proportion to the concentration of fly ash and length of exposure. An occasional exfoliated cell could be observed in the lumen of the explants. Because of the exfoliation, intercellular gaps were observed. Vacuolation was common in the apex of many epithelial cells. Morphological observations indicate that cytotoxic concentrations of oil-fired fly ash are below the level that produce ciliostasis.

TABLE 33

MEAN CILIA BEATING FREQUENCY IN HAMSTER TRACHEAL RING CULTURES AT VARIOUS INTERVALS DURING TEN 3 HR *IN VITRO* EXPOSURES TO FLY ASH FROM AN OIL-FIRED POWER PLANT

Fly Ash Concentration (µg/ml)	Mean Cilia Beating Frequency* Days in Culture								
	1	2	3	4	7	8	9	10	11
0	1264[a]	1258[a]	1257[a]	1228[a]	1200[a]	1208[a]	1201[a]	1190[a]	1147[a]
10	1210[b]	1195[b]	1221[a]	1175[b]	1135[b]	1115[b]	1153[b]	1101[b]	1083[b]
50	1140[c]	1165[b]	1127[b]	1114[c]	1087[c]	1110[b]	0[**]	0[**]	0[**]
100	1097[d]	1091[c]	1092[bc]	1102[c]	1075[c]	1110[b]	0[**]	0[**]	0[**]
250	1062[d]	1106[c]	1049[c]	0[**]	0[**]	0[**]	0[**]	0[**]	0[**]

Values with the same superscript within each column of a treatment are not significantly different ($P \leq 0.05$; Duncan's New Multiple Range Test).

*Mean of 16 observations of four tracheal explants.

**Not included in statistical analysis.

The standard error ranges which were <5% were omitted from the table for clarity.

TABLE 34

MEAN PERCENTAGE OF NORMAL EPITHELIAL LAYER IN HAMSTER TRACHEAL RING CULTURES AT VARIOUS INTERVALS DURING TEN 3 HR *IN VITRO* EXPOSURES TO FLY ASH FROM AN OIL-FIRED POWER PLANT

Fly Ash Concentration (µg/ml)	Mean Percent of Normal Epithelium* Days in Culture								
	1	2	3	4	7	8	9	10	11
0	97[a]	92[a]	93[a]	93[a]	86[a]	84[a]	73[a]	66[a]	56[a]
10	93[a]	90[a]	86[a]	81[b]	54[b]	45[b]	27[b]	20[b]	8[b]
50	85[b]	76[b]	63[b]	30[c]	13[c]	8[c]	0**	0**	0**
100	84[b]	60[c]	51[c]	33[c]	8[cd]	5[c]	0**	0**	0**
250	41[c]	24[d]	13[d]	0**	0**	0**	0**	0**	0**

Values with the same superscript within each column of a treatment are not significantly different($P \leq 0.05$; Chi-Square Distribition Test).

*Mean of four tracheal explants; Normal epithelium defined as a smooth luminal surface with beating cilia.

**Not included in statistical analysis.

The standard error ranges which were <7% were omitted from the table for clarity.

Effects of DFEE on Hamster Tracheal Ring Epithelium in Organ Culture

Studies were conducted to determine the concentration of DFEE that produces functional and morphological changes in organ cultures of tracheal mucosal epithelium. The material used in these studies was an Oldsmobile 350 diesel fuel exhaust particulate extract prepared by EPA. Originally, the extract was in methylene chloride at a concentration of 48.4 mg/ml. The methylene chloride was evaporated to dryness and resuspended in DMSO to a concentration of 100 mg/ml. DMSO controls and untreated controls were included.

Hamster tracheal ring explants were exposed *in vitro* to 10, 50 and 250 µg DFEE/ml of medium for 3 hr/day, 5 days/week for 2 weeks. Explants were exposed using a platform rocking at 10 cycles/min, causing the medium with DFEE or DMSO to flow continuously over the epithelial surface. Measurements of cilia beating frequency and cytopathology were determined on four cultures/observation and four cultures were fixed for histological examination.

Table 35 shows the mean cilia beating of ring explants following exposure to the various concentrations of DFEE and DMSO. The 0.01% DMSO control was similar to the untreated controls throughout the experiment. The means for the 0.05% DMSO control showed significant differences only at the 1, 3 and 8 day readings, while the cilia beating frequency of the tracheal ring cultures held in 0.25% DMSO declined significantly at days 2 and 9. The beating frequency in the 10 µg DFEE (0.01% DMSO/ml) treatment group was significantly different from its respective DMSO control at days 2, 4, 8, 9 and 10. A sharp decline at day 10 was observed compared to the previous days reading. The measurements in the 50 (0.05% DMSO) and 250 (0.25% DMSO) µg DFEE/ml were significantly lower than the respective untreated, DMSO controls group throughout almost all the experimental period.

Cytopathological examination of the tracheal mucosa showed significant alterations in the 0.01 and 0.05% DMSO controls beginning with day 2 (Table 36). Cultures treated with 0.01% DMSO were not significantly different from the untreated controls by day 7, while cultures treated with DMSO at 0.05% remained altered throughout the study after day 2. With the exception of the initial exposure, the 0.25% DMSO treatment was statistically different from the untreated controls. Treatment with 10 and 50 µg DFEE/ml showed similar results to their corresponding DMSO control. One exception was the 10 µg/ml group which showed a significant amount of damage to the epithelium <1 and 9 days after initial exposure.

Histological examinations of tracheal epithelium maintained for 2 to 8 days (2 to 6 exposures) in medium without DMSO or DFEE was similar to the epithelium of the intact hamster (Table 37). Areas of epithelial flattening was noted in some cultures. After 10 exposures, one-third of the untreated controls showed basal cell hyperplasia. In cultures exposed to 0.01, 0.05 and 0.25% DMSO, stratified metaplastic epithelium was observed after two exposures and persisted through the study. Stratified metaplastic epithelium was also observed in cultures exposed to 10 and 50 µg DFEE/ml. Evidence of epidermoid metaplasia was noted after six exposures in the 250 µg DFEE/ml treatment group and in the 50 and 250 µg DFEE/ml treatment group and in the 50 and 250 µg DFEE/ml after 10 exposures. This was found only in the experimental groups.

TABLE 35

MEAN CILIA BEATING FREQUENCY IN HAMSTER TRACHEAL RING CULTURES AT VARIOUS INTERVALS DURING NINE 3 HR *IN VITRO* EXPOSURES TO DFEE AND DMSO

Concentrations DMSO (%)	Concentrations DFEE (µg/ml)	Mean Cilia Beating Frequency* Days in Culture 1	2	3	4	7	8	9	10
0	0	1247^{a}	1255^{a}	1210^{a}	1217^{a}	1210^{a}	1212^{a}	1220^{a}	1146^{a}
0.01	0	1224^{a}	1247^{a}	1235^{a}	1203^{ab}	1183^{a}	1183^{a}	1202^{ab}	1153^{a}
0.05	0	1216^{a}	1209^{b}	1196^{ab}	1165^{c}	1182^{a}	1170^{a}	1189^{bc}	1176^{a}
0.25	0	1219^{a}	1224^{b}	1193^{ab}	1205^{a}	1173^{ab}	1176^{ab}	1143^{d}	1155^{a}
0.01	10	1216^{a}	1214^{bcd}	1192^{ab}	1168^{c}	1165^{ab}	1164^{b}	1121^{d}	1033^{c}
0.05	50	1190^{b}	1199^{c}	1185^{ab}	1183^{bc}	1151^{bc}	1140^{bc}	1105de	1090^{b}
0.25	250	1167^{c}	1176^{d}	1172^{b}	1104^{d}	1132^{c}	1124^{c}	1072^{e}	1077^{b}

Values with the same superscript within each column of a treatment are not significantly different ($P \leq 0.05$; Duncan's New Multiple Range Test).

*Mean of 16 observations of four tracheal explants.

The standard error ranges which were <4% were omitted from the table for clarity.

TABLE 36

PERCENTAGE OF NORMAL EPITHELIAL LAYER IN HAMSTER TRACHEAL RING CULTURES AT VARIOUS INTERVALS DURING NINE 3 HR *IN VITRO* EXPOSURES TO DFEE AND DMSO

Concentrations		Mean Percent of Normal Epithelium*							
		Days in Culture							
DMSO (%)	DFEE (µg/ml)	1	2	3	4	7	8	9	10
0	0	89^{a}	89^{a}	81^{a}	80^{a}	77^{a}	74^{a}	71^{a}	68^{a}
0.01	0	88^{a}	85^{a}	75^{b}	76^{b}	65^{b}	65^{a}	60^{a}	36^{b}
0.05	0	77^{a}	78^{a}	65^{c}	67^{b}	58^{b}	50^{b}	44^{b}	38^{b}
0.25	0	78^{a}	72^{ab}	59^{cd}	61^{b}	52^{b}	50^{b}	35^{bc}	30^{bc}
0.01	10	83^{a}	77^{a}	74^{ab}	73^{b}	55^{b}	57^{a}	34^{bc}	28^{c}
0.05	50	76^{ab}	74^{a}	73^{b}	70^{b}	55^{b}	50^{b}	40^{b}	24^{cd}
0.25	250	62^{b}	66^{b}	61^{c}	61^{b}	52^{b}	40^{b}	23^{c}	13^{d}

*Mean of four tracheal explants; Normal epithelium defined as a smooth luminal surface with beating cilia.

Values with the same superscript within each column of a treated are not significantly different ($P \leq 0.05$; Chi-Square Distribution Test).

The standard error ranges which were <6% were omitted from the table for clarity.

Table 37

EPITHELIA CHARACTERISTICS OF HAMSTER TRACHEAL RING ORGAN CULTURES
FOLLOWING TEN 3 HR EXPOSURES TO DIESEL EXHAUST AND DMSO

No. of Exposures	Control & Test Concentrations	Histological States of Epithelia in Tracheal Organ Cultures						
		Within Normal Limits[a]	Flattening of Ciliated & Mucous Cells	Desquamation of Superficial Cells[b]	Focal Basal Cell Hyperplasia[c]	Diffuse Basal Cell Hyperplasia[d]	Stratified Metaplastic Epithelium[e]	Epidermoid Metaplasia[f]
1	Untreated	5/5*						
	DMSO-0.01%	6/6						
	DMSO-0.05%	7/7						
	DMSO-0.25%	5/8	3/8					
	DFEE-10 µg/ml + 0.01% DMSO	3/5	2/5					
	DFEE-50 µg/ml + 0.05% DMSO	4/7	3/7					
	DFEE-250 µg/ml + 0.25% DMSO	2/7	2/7	1/7	2/7			
2	Untreated	5/8	3/8					
	DMSO-0.01%	2/7	3/7				2/7	
	DMSO-0.05%		4/7				3/7	
	DMSO-0.25%		3/8		1/8		4/8	
	DFEE-10 µg/ml + 0.01% DMSO		4/6				2/6	
	DFEE-50 µg/ml + 0.05% DMSO		4/8				4/8	
	DFEE-250 µg/ml + 0.25% DMSO		4/9				5/9	
6	Untreated	3/7	2/7				2/7	
	DMSO-0.01%	3/9	2/9	2/9			2/9	
	DMSO-0.05%		3/8			3/8	2/8	
	DMSO-0.25%		2/8		2/8	3/8	1/8	
	DFEE-10 µg/ml + 0.01% DMSO			2/7	3/7		2/7	
	DFEE-50 µg/ml + 0.05% DMSO			3/7			4/7	
	DFEE-250 µg/ml + 0.25% DMSO			1/7	2/7		2/7	2/7
10	Untreated	3/9	3/9		2/9	1/9		
	DMSO-0.01%		2/6	4/6				
	DMSO-0.05%		3/8	3/8			2/8	
	DMSO-0.25%		5/8				3/8	
	DFEE-10 µg/ml + 0.01% DMSO		2/7	3/7			2/7	
	DFEE-50 µg/ml + 0.05% DMSO		2/9			2/9	2/9	3/9
	DFEE-250 µg/ml + 0.25% DMSO		2/9	1/9		1/9	3/9	2/9

DMSO - Dimethylsulfoxide; DFEE = Diesel Fuel Exhaust Extract

[a] Normal limits defined as pseudostratified ciliated, columnar epithelium with a single layer of basal cells.

[b] Exfoliation of differentiated surface epithelial cells; over 50% of the epithelial surface.

[c] Small foci with 2-4 cell layers.

[d] Diffuse hyperplasia 2-4 cell layers of foci with more than 4 cell layers.

[e] Layered polygonal and cuboidal cells with flattened superficial cells.

[f] More than 10% of the normal epithelium is replaced by squamous-like cells.

* Nine explants were initially processed for histological examination. Loss during processing and technical imperfections in histological sections precluded evaluation of equal numbers of specimens at each exposure interval.
Number explants displaying type of epithelium listed/total number of explants examined.

Comparison of the results from cilia beating frequency and cytopathology indicates that both indices should be considered in assessing toxicity of the mucociliary epithelium. As shown in the results, a general concentration and number of exposures effect seem to exist. Exposure to DFEE caused a series of changes that also appear to be time-related.

Effects of Cigarette Smoke Condensate on Hamster Tracheal Ring Epithelium in Organ Culture

Hamster tracheal ring organ cultures were exposed *in vitro* to 1, 10, 100 and 500 µg CSC/ml, 3 hr/day, 5 days/week for a total of 10 days. The CSC, supplied by EPA, was dissolved in acetone. The percentage of acetone used in the CSC dilutions, starting with the lowest concentration was 0.002, 0.004, 0.04 and 0.2%. The cultures were observed daily, after CSC exposure. The method of exposure and parameters used to express cytotoxicity were as described for DFEE.

The data shown in Table 38 indicate that the mean cilia beating frequency of explants in the acetone controls were significantly different from untreated controls and the rate of decline was dose related. Cultures exposed to 1 and 10 µg CSC/ml showed a significantly greater decline than their corresponding acetone control at the 2, 3, 4 and 10 measurements. At the 100 µg CSC/level, a significant decline was observed at all observation points except the initial and final readings. Tracheal cultures exposed to 500 µg CSC/ml showed the greatest decline in beating frequency, producing total cessation of beating at day 9.

Cytopathological examinations of the tracheal mucosa showed significant alterations compared to nontreated controls in all acetone control and CSC treatment groups (Table 39). In the presence of 100 and 500 µg CSC/ml, large amounts of mucus and desquamated cells were observed along the luminal surface. The cytopathic effect increased depending on the concentration of CSC and duration of exposure. Acetone at 0.002, 0.004 and 0.04% produced only minor alterations, while 0.2% acetone produced a toxic action on the epithelium similar to 100 µg CSC/ml. In general, throughout the study, only the 100 µg CSC/ml at the latter exposure periods and 500 µg CSC/ml at the earlier exposure periods caused more severe damage to the trachea than its corresponding acetone control.

Untreated control cultures maintained a healthy columnar epithelium throughout the study (Table 40). In cultures exposed to acetone, flattening of the mucociliary epithelium could be observed after the initial exposure. After the second exposure the acetone treated explants showed areas of stratified metaplasia which progressed to epidermoid metaplasia on occasion. CSC-treated explants showed early signs of toxicity as evident by sloughing of superficial cells into the lumen. Stratification was seen after two exposures in all CSC concentration with the exception of the 500 µg/ml level where cell death appeared in one-half the cultures. Following six exposures, CSC was strongly cytotoxic at the 100 and 500 µg/ml level.

TABLE 38

MEAN CILIA BEATING FREQUENCY IN HAMSTER TRACHEAL RING CULTURES AT VARIOUS INTERVALS DURING NINE 3 HR *IN VITRO* EXPOSURES TO CSC AND ACETONE

Concentrations		Mean Cilia Beating Frequency*							
		Days in Culture							
Acetone (%)	CSC (µg/ml)	1	2	3	4	7	8	9	10
0	0	1252^{a}	1248^{a}	1234^{a}	1229^{a}	1208^{a}	1199^{a}	1195^{a}	1178^{a}
0.002	0	1220^{b}	1222^{a}	1213^{ab}	1189^{b}	1170^{b}	1162^{b}	1156^{b}	1173^{a}
0.004	0	1202^{bc}	1222^{a}	1189^{bc}	1183^{b}	1174^{b}	1165^{bc}	1153^{b}	1152^{a}
0.04	0	1196^{bc}	1182^{b}	1175^{c}	1161^{c}	1134^{cd}	1132^{d}	1135^{b}	1087^{b}
0.2	0	1173^{c}	1189^{bc}	1168^{cd}	1149^{cd}	1114^{de}	1118^{cde}	1106^{c}	1110^{b}
0.002	1	1206^{bc}	1145^{cd}	1152^{d}	1138^{de}	1166^{b}	1163^{b}	1159^{b}	1103^{b}
0.004	10	1191^{cd}	1164^{bc}	1173^{cd}	1157^{cd}	1149^{bc}	1142^{bc}	1144^{b}	1118^{b}
0.04	100	1193^{bc}	1148^{d}	1121^{e}	1124^{e}	1094^{e}	1077^{f}	1103^{e}	1085^{b}
0.2	500	1198^{bc}	1148^{d}	1113^{e}	1074^{f}	1063^{f}	1100^{ef}	0**	0**

Values with the same superscript within each column of a treatment are not significantly different ($P \leq 0.05$; Duncan's New Multiple Range Test).

*Mean of 16 observations of four tracheal explants.

**Not included in statistical analysis.

The standard error ranges which were <5% were omitted from the table for clarity.

TABLE 39

PERCENTAGE OF NORMAL EPITHELIAL LAYER IN HAMSTER TRACHEAL RING CULTURES AT VARIOUS INTERVALS DURING NINE 3 HR *IN VITRO* EXPOSURES TO CSC AND ACETONE

Concentrations		Mean Percent of Normal Epithelium*							
		Days in Culture							
Acetone (%)	CSC (µg/ml)	1	2	3	4	7	8	9	10
0	0	94^{a}	98^{a}	94^{a}	94^{a}	90^{a}	89^{a}	86^{a}	79^{a}
0.002	0	85^{b}	80^{bc}	78^{b}	75^{bc}	70^{b}	66^{b}	60^{c}	45^{c}
0.004	0	89^{ab}	90^{ab}	78^{b}	78^{b}	71^{b}	71^{b}	69^{bc}	59^{b}
0.04	0	95^{a}	89^{ab}	66^{bc}	63^{c}	68^{b}	68^{b}	64^{c}	63^{b}
0.2	0	80^{b}	83^{b}	60^{c}	60^{c}	45^{c}	45^{c}	40^{d}	5^{ef}
0.002	1	84^{b}	83^{b}	70^{bc}	0^{bc}	78^{b}	78^{ab}	75^{ab}	60^{b}
0.004	10	84^{b}	76^{c}	70^{bc}	68^{bc}	63^{b}	63^{b}	59^{c}	43^{c}
0.04	100	90^{ab}	85^{b}	68^{c}	60^{c}	20^{d}	20^{d}	20^{e}	14^{de}
0.02	500	53^{c}	55^{d}	34^{d}	43^{d}	43^{c}	45^{c}	0**	0**

*Mean of four tracheal explants; Normal epithelium defined as a smooth luminal surface with beating cilia.

Values with the same superscript within each column of a treatment are not significantly different ($P \leq 0.05$; Chi-Square Distribution Test).

**Not included in statistical anlaysis.

The standard error ranges which were <6% were omitted from the table for clarity.

Table 40

EPITHELIA CHARACTERISTICS OF HAMSTER TRACHEAL RING ORGAN CULTURES
FOLLOWING NINE 3 HR EXPOSURES TO CSC AND ACETONE

No. of Exposures	Control & Test Concentrations	Histological States of Epithelia in Tracheal Organ Cultures: Within Normal Limits[a]	Flattening of Ciliated & Mucous Cells	Desquamation of Superficial Cells[b]	Focal Basal Cell Hyperplasia[c]	Diffuse Basal Cell Hyperplasia[d]	Stratified Metaplastic Epithelium[e]	Epidermoid Metaplasia[f]
1	Untreated	4/4*						
	Acetone-0.001%	2/4	2/4					
	Acetone-0.004%	2/4	2/4					
	Acetone-0.04%	2/4	2/4					
	Acetone-0.2%		2/4	1/4	1/4			
	CSC-1 µg/ml + 0.001% Acetone			2/4	2/4			
	CSC-10 µg/ml + 0.004% Acetone		3/4	1/4				
	CSC-100 µg/ml + 0.04% Acetone		1/4	3/4				
	CSC-500 µg/ml + 0.4% Acetone		1/4	3/4				
2	Untreated	2/4	1/4		1/4			
	Acetone-0.001%		3/3					
	Acetone-0.004%		2/2					
	Acetone-0.04%		1/4	1/4	1/4		1/4	
	Acetone-0.2%		1/4	2/4			1/4	
	CSC-1 µg/ml + 0.001% Acetone		2/4				2/4	
	CSC-10 µg/ml + 0.004% Acetone		1/4				2/4	1/4
	CSC-100 µg/ml + 0.04% Acetone		2/4				2/4	
	CSC-500 µg/ml + 0.2% Acetone	-cell death- 2 rings		2/2				
6	Untreated	1/4	1/4		2/4			
	Acetone-0.001%		1/4	1/4			1/4	1/4
	Acetone-0.004%			1/4	1/4		2/4	
	Acetone 0.04%			1/4			2/4	1/4
	Acetone-0.2%			1/4	1/4		1/4	1/4
	CSC-1 µg/ml + 0.001% Acetone		1/4		2/4		1/4	
	CSC-10 µg/ml + 0.004% Acetone		1/4		1/4		1/4	1/4
	CSC-100 µg/ml + 0.04% Acetone		1/2		-cell death- 2 rings			1/2
	CSC-500 µg/ml + 0.2% Acetone	-cell death- 3 rings						1/1
10	Untreated	2/4	1/4	1/4				
	Acetone-0.001%		1/4	1/4	1/4		1/4	
	Acetone-0.004%		2/4		1/4		1/4	
	Acetone-0.04%		1/4	1/4			1/4	1/4
	Acetone-0.2%		2/3		-cell death- 1 ring			1/3
	CSC-1 µg/ml + 0.001% Acetone		2/4	1/4	1/4			
	CSC-10 µg/ml + 0.004% Acetone					3/4	1/4	
	CSC-100 µg/ml + 0.04% Acetone		1/4				2/4	1/4
	CSC-500 µg/ml + 0.2% Acetone	-cell death- 4 rings						

CSC = cigarette smoke condensate

[a] Normal limits defined as pseudostratified ciliated, columnar epithelium with a single layer of basal cells.

[b] Exfoliation of differentiated surface epithelial cells; over 50% of the epithelial surface.

[c] Small foci with 2-4 cell layers.

[d] Diffuse hyperplasia 2-4 cell layers of foci with more than 4 cell layers.

[e] Layered polygonal and cuboidal cells with flattened superficial cells.

[f] More than 10% of the normal epithelium is replaced by squamous-like cells.

* Nine explants were initially processed for histological examination. Loss during processing and technical imperfections in histological sections precluded evaluation of equal numbers of specimens at each exposure interval.
Number explants displaying type of epithelium listed/total number of explants examined.

The work reported here has demonstrated that the epithelium of hamster trachea in organ culture respond to complex real-world particulate pollutants in a manner that can be interpreted in terms of cytotoxicity or changes in the pattern of differentiation. The toxic effects produced by the two fly ash species were the result of different mechanisms of inducing injury. Morphological observations indicated that cytotoxic concentrations of oil-fired fly ash were below the level that produce ciliostasis. The transformation of tracheobronchial epithelium to epidermoid metaplasia by coal-fired fly ash shows similiarities in response to mechanical stimuli[18], effects of carcinogens[19-21], vitamin A deficiency[22], and nutritional influences *in vitro*[23]. The effects of CSC and DFEE on ciliated columnar epithelium in culture were comparable to the response of coal-fired fly ash. The sequence of changes illicited by these pollutants following a series of daily 1 or 3 hr exposures was roughly proportional to the concentration and length of exposure.

Our model system whose responses to toxic materials are easily measured, appear to serve as a tissue-specific assay for assessing effects of complex chemical entering our environment. *In vitro* exposure of tracheal explants to fly ash responded in a similar manner to tracheobronchial epithelium of hamster exposed *in vivo*, indicating its usefulness as a bioassay.

The significance of the changes observed in the tracheal organ cultures described in our studies must be evaluated not only in terms of toxicity but also carcinogenicity. The occurrence of epidermoid metaplasia with extensive keratin formation in suckling hamster trachea in organ culture demonstrates a greater responsiveness of immature respiratory tissue compared to ring cultures from adult animals. We have not established whether the cytological alterations observed constitute preneoplastic events. Known carcinogenic polycyclic hydrocarbons when added to organ culture medium have produced similar abnormal histologic states in respiratory epithelium of suckling hamsters and rats[24-26].

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